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COMPARATIVE STUDIES ON THE STRUCTURE OF HUMAN  
ADENOVIRUS GENOMES 4 7 AND 21(U) MARYLAND UNIV  
BALTIMORE DEPT OF BIOLOGICAL CHEMISTRY R PADMANABHAN

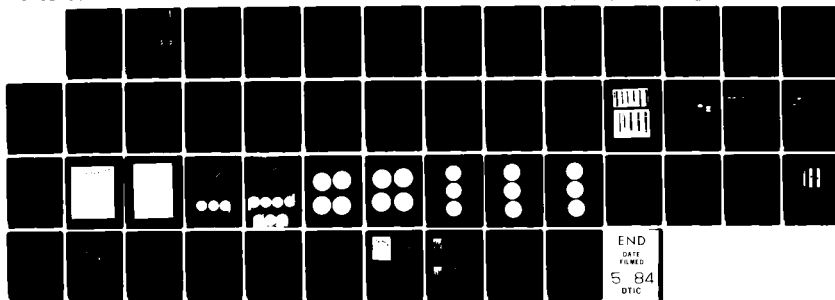
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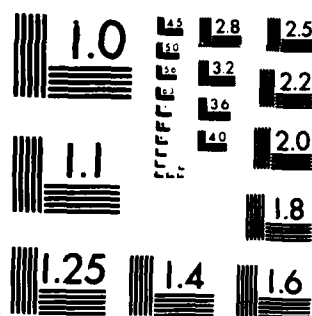
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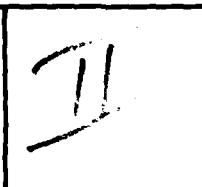




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'Comparative Studies on the Structure of Human Adenovirus Genomes 4, 7 and 21'

Annual Progress Report

by

Radhakrishnan Padmanabhan, Ph.D.

Annual Progress Report

February 1981

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## Abstract

1. A sensitive method was developed in our laboratory to label the 5' termini of Ad DNA which was found to be applicable to Ad 7, Ad 4 and Ad 21 DNA due to the presence of a tyrosine-containing peptide covalently attached to these DNA molecules. We used this method to map the cleavage sites of Ad 7 (Greider) DNA with ten restriction endonucleases (EcoRI, Hind III, Bam HI, BclI, BstEII, XbaI, SmaI, HpaI, Sall, KpnI).
2. Ad 7 vaccine strain was passaged in 293 cells and then grown in large amounts in suspension cultures of KB cells. The DNA was extracted and purified. Restriction enzyme analysis of vaccine and Greider Ad 7 strains revealed that the two strains gave identical cleavage patterns with 8 restriction enzymes.
3. Ad 4 prototype strain (ATCC) have been passaged in 293 cells and then grown in large amounts in suspension cultures of human KB cells. The DNA from these virions have been extracted and purified. Using our sensitive method of terminal labeling, we have mapped the terminal restriction fragments of Ad 4 (ATCC strain) with several restriction enzymes. We are currently mapping the cleavage sites of these enzyme on the DNA. Once this is completed, Ad 4 DNA from the vaccine strain will be analyzed by a similar manner.
4. Ad 21 vaccine strain has been passaged in 293 cells and then the virus was grown in KB cell suspension cultures. We are currently in the process of isolating the DNA and analyzing its genomic organization with several restriction enzymes.
5. We analyzed the efficiencies of different protocols for titering the live Ad vaccine strains Ad 4, Ad 7 and Ad 21 present in enteric coated tablets. We used HeLa cells, 293 (human embryonic kidney cells transformed by Ad 5), KB cells and 549 cells as the host. We found that 293 cells are the most suitable for the plaque assay of these viruses. We compared different protocols for the overlay medium used in the titration procedure such as the high  $Mg^{2+}$  concentration (Williams, 1970), dimethyl sulfoxide alone and dimethyl sulfoxide in the presence of 0.01% DEAE-dextran (McCown et al., 1979). From our studies, it is evident that a combination of 1% DMSO and 0.01% DEAE-dextran is the most effective in enhancing the plaque formation in 293 cells. We have recently obtained evidence that an overlay containing 1% DMSO and 0.01% DEAE-dextran enhances the transfection of Ad 7 DNA into 293 cells.

## General Introduction

Human adenoviruses have been classified under DNA Tumor Virus group since they produce tumor in newborn hamsters. However, there is no evidence so far to link them as causative agents in human cancers. All human adenoviruses, except probably Ad 4 (since the data for Ad 4 is lacking), can transform rat embryo cells in vitro and the transformed cells are tumorigenic when injected to newborn hamsters or rats. All early experiments on in vitro transformation were conducted with cells of non-human origin such as rat embryo, and the difficulty of using human cells was due to the permissiveness of the cell for the replication of the virus. However, it was shown by Graham et al. (1) that sheared Ad 5 DNA could transform human embryonic kidney cells in vitro. The resulting transformed cells (293 cells) are now used for isolating mutants of Ad 5 defective in transformation (2). Integrated genes from the left-end of Ad 5 DNA present in 293 cells are able to complement the defective genes for transformation in the mutant. Such mutants could not replicate in HeLa or human KB cells.

Due to the involvement of human adenoviruses 4, 7 and 21 in the Acute Respiratory Diseases in military recruits and civilian populations, live vaccines against these viruses have been developed (3,4) which seemed to control this disease.

The central goal of the original research proposal is (1) to study the genome structure of these viruses which have been developed as vaccines (Ad 4, 7 and 21), using several restriction enzymes. The data will facilitate our future investigation of the physical organization of various genes, controlling the important functions such as DNA replication, transformation and perhaps oncogenicity, the region coding for the synthesis of viral type-specific and group-specific antigens in the life cycle of the virus. (2) We propose to generate mutants of adenovirus which are defective in transformation and oncogenicity. The region essential for transformation has been localized on the Ad genome to be at the left-end of the DNA between 4-16%. This part of the viral DNA gets integrated into the host genome during cell transformation and the cells are permanently transformed. Using Ad 5-transformed human embryonic kidney cells as host, it has been possible to produce transformation-defective Ad 5 mutants by in vitro manipulation of Ad 5 DNA. These studies raised new questions such as whether Ad 5-transformed HEK cells could function as host for transformation defective, Ad 7 or Ad 21 mutants; whether these mutants are oncogenic in newborn hamsters.

Original contract proposal which has been in effect since July 1, 1979 was awarded for the comparative studies of the genomes of Ads 4, 7 and 21. The personnel supported by the contract, their experience and their efforts expended during the duration of the contract since February 1980 - present are given below.

Personnel	Experience	Period Supported by contract.
Osamu Tokunaga M.D., Ph.D.	Tissue culture and tumor biology	January 1980 - present
Ms. Monica Buescher	Medical Student; three years experience in molecular biology during her undergraduate curriculum at J.H.U., Baltimore, MD.	month of August 1980 and Jan. 1981. She won a Dean's summer fellowship for June and July 1980.
Ms. Karen Turner	Lab. Assistant (part-time; B.S. degree holder. Learning tissue culture and virology	Dec. 15, 1980 - present.
David Weber	Lab. Scientist I (temporary). Finished all his requirements towards M.D. degree.	Jan. 5 - March 31, 1981.
Sheila Bond, Ph.D.	Tissue Culture and Virology	Jan. 5 - present.
Morikazu Shinagawa D.V.M., Ph.D. Visiting Scientist Currently Assistant Professor, Obihiro University School of Medicine, Obihiro Japan.	Virology and Nucleic Acid Structure Since 1977	March - April 30, 1981.
Radha K. Padmanabhan Principal Investigator University of Maryland School of Medicine	Virology and Nucleic Acid Structure and Function, Since 1969.	July 1, 1980 - August 30, 1980.

The progress supported by this contract has resulted in the submission or preparation of the following manuscripts. Five complete sets with original figures are included and less satisfactory photocopies are included for the remainder of the required copies. If additional copies are required, they will be submitted as soon as possible.



#### PUBLICATIONS (Since Feb. 2, 1980).

1. Roninson, I. and Padmanabhan, R. (1980) Studies on the nature of the linkage between the terminal protein and the adenovirus DNA. Biochem. Biophys. Res. Commun. 94, 398-405.
2. Shinagawa, M. and Padmanabhan, R. (1980) comparative sequence analysis of the inverted terminal repetitions from different adenoviruses. Proc. Natl. Acad. Sci. 77, 3831-3835.

#### Manuscripts in Preparation

1. Buescher, M., Roninson, I. and Padmanabhan, R. (1980) Physical Mapping of Adenovirus DNA by a Simple and Sensitive Method of Terminal Labeling.
2. Tokunaga, O., Padmanabhan, R., Scott, R.M. and Bancroft, W.H. (1980) An Improved Plaque Assay for Adenovirus Vaccine Strains.

#### Physical Mapping of Ad 7 (Greider) Strain and Ad 7 Vaccine Strain.

In the last Progress Report, we described a method to label the 5' termini of Ad 7 DNA (which was also found to be applicable to Ad 2 DNA) due to the presence of a covalently attached, tyrosine - containing peptide at each terminus. We demonstrated the usefulness of this terminal labeling method to physically map Ad 7 DNA for the cleavage sites of BclI. During the period covered by this report, we mapped the restriction sites of Ad 7 (Greider) with 10 different enzymes using the simple and sensitive labeling method.

#### Methodology

We used the method of partial hydrolysis of a single end labeled DNA fragment (see Ref. 5) by a restriction enzyme (Smith and Birnsteil, 1976). We confirmed the map units of the cleavage sites independently by mixed hydrolysis with a second enzyme.

#### Results

For physical mapping using the method of partial hydrolysis of Smith and Birnsteil (1976), it is required to have the DNA fragment labeled at a single terminus. This is achieved in Ad 7 DNA (Greider) due to the presence of a single EcoRI site. Cleavage of Ad 7 DNA labeled at 5' termini with EcoRI produces EcoRI-A (86%) and EcoRI-B each containing [<sup>125</sup>I] label at its 5' terminus. [<sup>125</sup>I] labeled A and B fragments were fractionated on a 5-20% gradient of sucrose, centrifuged in a SW41 rotor (Beckman Instruments Inc. Palo Alto, Calif. USA) at 35,000 rpm for 7 hr. The fractions in 250 µl aliquots were collected and counted in a gamma counter (Packard Instruments). The EcoRI-A and B fragments were, dialyzed against TE buffer and precipitated with ethanol. The DNA fragments were dissolved in TE buffer for further digestion with other restriction enzymes.

#### Sizes of the terminal fragments generated by each restriction enzyme.

[<sup>125</sup>I] labeled Ad 7 DNA was digested with various restriction enzymes (see Fig. 1) - BamHI (a & b), KpnI (c & d), Sall (e & f), HpaI (g & h), EcoRI (i & j), BclI (k & l), SmaI (m & n), XbaI (o & p), XhoI (q & r), HindIII (s & t), and BstII (u & v). The ethidium bromide fluorescence of DNA fragments was photographed and [<sup>125</sup>I] radioactivity was detected

by autoradiography. From these data, it was possible to calculate the sizes of the DNA fragments generated by each restriction endonuclease and in addition, to detect the labeled terminal fragments (Fig. 1 and Table I). By cleaving the labeled EcoRI-A and B fragments with each restriction endonuclease, it was possible to deduce the orientation of the terminal fragments with respect to the right or left end of the viral genome.

The order of the various DNA fragments generated by each restriction endonuclease was deduced by using the technique of partial hydrolysis of a single end labeled DNA fragment with a restriction enzyme (Smith and Birnstein, 1976; Jay and Wu, 1976). In some cases the physical map was constructed in conjunction with mixed hydrolysis of Ad 7 with two restriction endonucleases, the cleavage sites of one of which had already been deduced unambiguously.

#### Cleavage map of Ad 7-EcoRI (Fig. 1, i & j).

We confirmed the earlier finding of Sekikawa and Fujinaga (8) that there is only one EcoRI site which mapped at 86.0% from the left end of Ad 7 (Greider) DNA. This property enabled us to separate the two fragments readily by velocity sedimentation on gradients of sucrose.

#### Cleavage with Sall (Fig. 1e & 1f).

Cleavage of EcoRI-A with Sall showed that Sall-C is from the left-end of the genome and therefore, the linear order of Sall fragments in Ad 7 (Greider) is CAB.

#### Cleavage with HpaI (Fig. 1g & 1h).

Knowing that HpaI-A fragment comes from the left end and HpaI-C from the right end, the linear order can be deduced as A-B-C.

#### Cleavage with BclI.

Cleavage of Ad 7 with BclI produced eight fragments, of which BclI-A was derived from the left terminus and D was from the right (Fig. 1k and l). For restriction site mapping analysis, either native [<sup>125</sup>I] labeled Ad 7 DNA or (EcoRI-A & B fragments) were used. The terminal fragments (A and D) are so different in their sizes that the labeled fragments resulting from a partial hydrolysis were separable from each other. Fig. 3 c & d shows the autoradiography of the gel. From the sizes of the partial restriction products fused to the left-end of the genome, the linear order was deduced as A-E-C-G (see Table II). Similar estimation of the sizes of fragments at the right-end of the genome gave rise to an order of D-F-H. By the process of elimination, BclI-B was placed between BclI-G and H fragments to give the linear order of A-E-C-G-B-H-F-D.

This order was confirmed by digesting <sup>32</sup>-[P] labeled EcoRI-A and B fragments partially with BclI. EcoRI-B includes the terminal BclI-D (9.4%) and a portion (4.4%) of BclI-F fragment. Ad 7 was cleaved with EcoRI, dephosphorylated by treatment with phosphatase and labeled at the 5' termini using polynucleotide kinase and [ $\gamma$ -<sup>32</sup>P] ATP. Under these conditions, the native 5' termini were blocked (Carusi, 1977) and only the EcoRI-termini were labeled. The sizes of the partial digestion products of EcoRI-A and B fragments with BclI are consistent with the linear order given above. As a further confirmation, we digested Ad 7 with a combination of two restriction enzymes BamHI + BclI and BclI + KpnI. The sizes of the DNA fragments obtained were determined (Table III). These values are consistent with the linear order derived from partial hydrolysis of [<sup>125</sup>I] labeled Ad 7 DNA.

#### Cleavage with SmaI.

Ad 7 digested with SmaI yielded nine fragments, of which fragments D and I from the left and right termini, respectively (Fig. 1). The sizes of the fragments are given in Table I.

The SmaI cleavage sites were mapped on the Ad 7 genome by electrophoretic analysis of DNA fragments obtained by (1) mixed hydrolysis of the whole genome with SmaI and HpaI (Table III) and (2) partial hydrolysis of terminal [<sup>125</sup>I] labeled EcoRI-A with SmaI. Data shown in Fig. 2 and Table V indicate that the two HpaI cleavage sites mapped at 69.3% and 90.0% (see above) are located in SmaI-E and SmaI-B fragments respectively. Four new fragments, 12%, 8.8%, 6.7% and 1.7% were generated (Table III). HpaI site at 90.0% is located in either SmaI-B or E fragment. SmaI cleavage site at the right hand terminus is located at 97.0% and if E fragment is adjacent to I (between 86.2%-97.0% of the genome) then HpaI cleavage at 90.0% would have produced two new fragments with sizes of 3.8% and 7.0%. However, if SmaI-B fragment is located adjacent to SmaI-I (between 78.4% - 97.0%), then HpaI cleavage at 90% would be expected to generate two new fragments, 11.6% and 7.0% in length. These fragments are indeed generated as shown in Table III. Hence, SmaI-E fragment located between 67.6 - 78.4 MU is cleaved by HpaI generating at 69.3 MU two new fragments, 8.8% and a 1.7% in length.

Partial hydrolysis of single end-[<sup>125</sup>I]-labeled, EcoRI-A with SmaI gave rise to a series of fragments fused to the terminal SmaI-D fragment from the left-end. The estimated sizes of these fragments are shown in Table V. The linear order from the left end deduced is D-H-G-A-C. The size difference between fused fragments b and a is 16.1% which is closer to SmaI-B than C. However, the location of SmaI-B is already established as adjacent to the right-terminal I fragment. Hence, C fragment is placed adjacent to A. From these data, the map could be deduced only upto 60.9% from the left-end and 78.4%-100 at the right end (SmaI-B + I). Knowing that HpaI cleaves SmaI-E fragment at 69.3 to produce two fragments of 8.8% and 1.7%, the linear order of D-H-G-A-C-F-E-B-I is deduced for SmaI cleavage sites.

#### Cleavage with KpnI.

Ad 7 digested with KpnI produced four fragments, of which A is from the left and B is from the right terminus (see Fig. 1). The order of C and D were established by a mixed hydrolysis of Ad 7 with BclI and KpnI (Table III). The linear order deduced for KpnI cleavage sites is A-D-C-B.

#### Cleavage with BamHI.

Ad 7 digested with BamHI yielded 10 fragments, of which J and F are the left and right terminal fragments, respectively. The BamHI sites were mapped on the Ad 7 genome by electrophoretic analysis of DNA fragments obtained by (1) partial hydrolysis of single end-[<sup>125</sup>I] or [<sup>32</sup>P]-labeled EcoRI-A or B with BamHI (Fig. 2 and Table IV) and (2) mixed hydrolysis of the whole genome with BclI and BamHI (Table III) (3) partial hydrolysis of whole Ad 7 DNA labeled with [<sup>125</sup>I] (Fig. 4 c & d).

The data shown in Table IV gives the linear physical order from the [<sup>125</sup>I]-labeled left end as J-G-H-I-A. Partial hydrolysis of [<sup>125</sup>I]-EcoRI-B (Fig. 2C) produced BamHI-F (7.4%) as only terminally labeled fragment. Ethidium bromide fluorescence showed two new bands with the estimated sizes of 6.2% and 4.0%, the sum of which correspond to the size of BamHI D. Hence, BamHI D is adjacent to the right-terminal BamHI-F fragment. The fact that EcoRI site located in BamHI-D is confirmed by the data shown in Table IV. In this experiment, EcoRI-A and B fragments were labeled at the 5' termini of EcoRI site exclusively, taking advantage of the fact that the native 5' termini are blocked to phosphorylation (Carusi, 1977). Two 5' labeled fragments (D<sub>1</sub> + D<sub>2</sub>) (Table IV), with the sum of 10.2% of the genome represent BamHI-D cleaved by EcoRI (We have, arbitrarily placed BamHI-D as the fragment cleaved by EcoRI). The linear order for BamHI fragments from the EcoRI site towards the left terminus is D<sub>2</sub>-C-E-B and towards the right terminus is D<sub>1</sub>-F. From these data, the linear order deduced for BamHI cleavage sites from left to right is J-G-H-I-A-B-E-C-D-F. The physical map deduced for BamHI is consistent with the pattern obtained by a mixed hydrolysis of Ad 7 with BamHI + BclI and BamHI + Sall (Table III).

#### Cleavage with BstEII.

Cleavage of Ad 7 with BstEII yields eight fragments, of which fragments A and H represent the left and right termini, respectively (Fig. 1). Cleavage of EcoRI-A fragment with BstEII produced fragments A,B,E,F and G, 0.4% of the genome clipped off from C. Since D and H were missing, they must come from EcoRI-B fragment (data not shown). These data established the order H-D-C . . . near the right terminus. Partial hydrolysis of [<sup>125</sup>I] labeled Ad 7 DNA (Fig. 4a & b) or [<sup>125</sup>I] EcoRI-A (Fig. 3 a & b) with BstEII produced labeled fragments of estimated sizes as shown in Table V. The addition of F to the left-terminal A produced a size difference of 5.9% which is very close to the size of BstEII-F or G fragment. This resulted in an ambiguity of F or G adjacent to A and it is essentially due to the difficulty of determining the sizes accurately in this range. This was solved by cleaving Ad 7 with a mixture of BamHI and BstEII. The sizes of the fragments generated are given in Table III which is consistent with the assignment of F adjacent to A. The linear order of DNA fragments deduced from the data shown in Table V is A-F-E-B-G-C-D-H.

#### Cleavage with XbaI.

Cleavage of Ad 7 with XbaI yields four fragments, of which fragments B and C were the terminal fragments (Fig. 1). The orientation of the left and right terminal fragments was established by digesting [<sup>125</sup>I]-EcoRI-A fragment with XbaI. Labeled XbaI-B was produced and XbaI-C was missing indicating that B comes from the left-end and C from the right end. A new DNA fragment with a size of 6% was produced consistent with the difference in size between EcoRI-B (14%) and XbaI-C (20%). The physical map of XbaI cleavage sites was established from a double digest with KpnI. The sizes of the fragments produced in a complete digest with XbaI alone (Table I) and XbaI + KpnI (Table III) are shown. The linear order consistent with these data is B-D-A-C. The composite physical map of Ad 7 DNA (Greider) strain indicating the cleavage sites of 10 restriction enzymes is given in Fig. 5.

### 2. Restriction enzyme analysis of Ad 7 vaccine strain and comparison with Ad 7 (Greider) strain

#### Methodology

Live Ad 7 vaccine strain from enteric coated vaccine tablets were passaged in HEK cells transformed by Ad 5 (293 cells). The virus stock prepared in this manner was used to infect KB cells in suspension cultures. The virus was released by sonication and purified by CsCl equilibrium density gradient centrifugation. The DNA was extracted from the virions by treatment with SDS, protease K and phenol. The DNA was dialyzed against 10mm Tris-HCl, pH 7.4 and 1mm EDTA and precipitated with ethanol. The precipitate was dissolved in 10mm Tris-HCl, pH 7.4 and 1mm EDTA and used for restriction enzyme analysis.

#### Results

Fig. 6 shows the comparison of the cleavage patterns of Ad 7 (Greider) and vaccine strains. The two DNAs were digested with 8 restriction enzymes, EcoRI, SalI, XbaI, BamHI, BclI, BstEII, HindIII and SmaI. The two strains exhibited identical cleavage patterns.

### Significance

From these data, it is evident that classification of an Ad strain by the analysis of its genome for the cleavage sites of restriction enzymes is an alternate but powerful approach compared to the conventional techniques based on serological properties. The data on the physical map of Ad 7 are the prerequisites for the isolation of transformation-defective mutants.

Wadell and Varsanyi (1978) (see Ref. 9) reported an interesting subgrouping of Ad 7 strains based on their differences in their restriction enzyme cleavage patterns. Eight strains, all classified as Ad 7 from their immunological properties, were analyzed for their cleavage patterns with six enzymes; EcoRI, HpaI, SmaI, HindIII, Sall and BamHI. Their cleavage patterns fall into three groups. The prototype Ad 7 (Gomen) belongs to one, as its cleavage patterns are distinctly different from either of the other two groups. Strains 1058, 55142 (vaccine strain, obtained from M. Rosenbaum, Rockford, Ill.) and 1380 belong to the second group (Ad 7a subtype). Strains 879, 952, 860 and 211 belong to the third group (Ad 7b subtype).

We compared the cleavage patterns of the Ad 7 vaccine strain, isolated from the enteric coated tablets (Lot. No. 8001) with those of the strains from these three groups. We found that the cleavage patterns of Ad 7 vaccine strain were found to be identical to those in Ad 7a subtype for five out of six restriction enzymes, used by Wadell & Varsanyi, (1978). The only difference is the EcoRI pattern of Ad 7 vaccine strain (Lot. No. 8001) which has only one site compared to that of strain 55,142 in Ad 7a Subtype which has two EcoRI sites. We cannot explain the difference in the EcoRI pattern exhibited by the vaccine strain (55,142) analyzed by Wadell and Varsanyi and the strain rescued from the live enteric coated vaccine tablets by us. One plausible explanation is that Ad 7 vaccine strain (Lot. No. 8001) lost one EcoRI site due to mutation, resulting from several serial passages. This (85.4 MU) which might result in a difference in hemagglutination properties between these two strains. In spite of the minor difference in the EcoRI cleavage pattern, our data on the cleavage analysis of the vaccine strain and comparison with the known patterns of other Ad 7 strains, show that Ad 7 vaccine strain 1) belongs to Ad 7a subtype 2) exhibits identical cleavage patterns of Ad 7 (Greider strain) which have been mapped for ten restriction endonuclease recognition sites. The data on the detailed physical map of these strains will be helpful to monitor any mutational changes (such as substitution or deletion in their genomes) occurring in the future manufactured lots of vaccine strain.

### 3. Restriction enzyme analysis of Ad 4 prototype (ATCC VR-4, LOT 6) and vaccine strain (Lot. 7901).

Ad 4 prototype strain (ATCC, VR-4) has been passaged in HEK cells transformed by Ad 5 (293 cells) and a high titer stock has been prepared. This stock was used to infect human KB cells in suspension cultures to prepare the virus in quantities sufficient to analyze its genomic organization. We analyzed the cleavage pattern of Ad 4 with the restriction enzymes, BclI, HindIII, Sall, EcoRI, XbaI, BamHI, BstEII and SmaI. The cleavage patterns are shown in Fig. 7.

We labeled the Ad 4 DNA with [<sup>125</sup>I] using the sensitive method discovered in our laboratory. (10) Currently, we are mapping the cleavage sites of these restriction enzymes on Ad 4 DNA. We have also passaged the Ad 4 vaccine strain in 293 cells to prepare a high titer stock of this virus. This stock will be used to infect human KB cells in suspension cultures and the virus in sufficient quantity will be prepared for analysis of its genomic organization.

#### 4. Restriction enzyme analysis of Ad 21 vaccine strain

Ad 21 vaccine strain (obtained from Drs. R.M. Scott and W.H. Bancroft) has been passaged in 293 cells and a high titer stock has been prepared. We are currently using this stock to infect human KB cells in suspension cultures to prepare the virus in sufficient quantities for the isolation and purification of its DNA. We will analyze its genomic structure during the remaining part of the current contract period.

#### 5. Infectivity Assays of Adenovirus vaccine strains

We proposed in the original application to establish the optimum conditions for assaying the infectivity of Ad strains in the live enteric coated tablets. These data will augment the existing methodology of titering the virus by hemagglutination-inhibition assays, the neutralization test, the complement fixation test, the fluorescent antibody test and the radioimmune assay. We compared the host range of these vaccine strains, using HeLa, KB, 293 (HEK-transformed by Ad 5) and 549 cells. We also compared different protocols for titration, such as the agar overlay containing high  $MgCl_2$  concentration (Williams, 1970) and the overlay containing dimethyl sulfoxide (DMSO) and DEAE-dextran (McCown et al., 1979) originally developed to assay phlebotomus fever virus. The optimum system to titer the live Ad vaccine strains present in enteric coated tablets (without further serial passage into any permissive host) is the use of 293 cells as the host and the agar overlay containing DMSO (1%) + 0.01% DEAE-dextran.

#### Methodology

The enteric coated vaccine tablets of Ad 4 (Lot. No. 7901) and Ad 7 (Lot. No. 08001 and 08201) were obtained from Drs. R.M. Scott and W.H. Bancroft (Walter Reed Army Institute of Research, Washington, D.C.). Five tablets of each strain were used to prepare the virus suspension. The tablets were cracked in a porcelain mortar and suspended as a fine powder in 20 ml of MEM supplemented with 5% gamma globulin-free calf serum (Gibco). The solution was centrifuged down at 800 rpm for 5 min. in an IEC centrifuge and the supernatant was recovered. It was filtered through a 0.44 $\mu$ m membrane filter. 5 ml of 50% glycerol was added to 20 ml of virus supernatant solution and this mixture was stored in aliquots at -70°C freezer (Revco).

HEK cells transformed by Ad 5 (Graham et al.) and 549 cells were obtained from Dr. R.M. Scott (Walter Reed Army Institute of Research), HeLa cells and KB cells from Dr. J. Rose (NIAID, N.I.H.). These cells were maintained in MEM (Eagle) supplemented with 5% calf serum and standard concentrations of penicillin-streptomycin, glutamine and sodium bicarbonate (Flow Labs. MD) in a CO<sub>2</sub> incubator (5%) at 37°C. The cells were infected when the cell monolayer was about 70% confluent.

Before studying the various parameters in a plaque assay system, it was necessary to determine the time required for the development of cytopathogenic effect (CPE) in these cells by the infection of Ad vaccine strains. Ad 4 strain showed CPE in 293 cells on day 6, whereas Ad 7 vaccine strain showed CPE on day 16, but both Ads did not show any CPE in KB or HeLa cells up to 18 days. However, both Ad 4 and 7 vaccine strains rescued from enteric coated vaccine tablets regained their ability to show CPE in HeLa and KB cells after their serial passages in 293 cells. This serially passaged Ad 4 and 7 vaccine strain could show CPE in HeLa and KB cells on day 2. These data suggest that the relative adsorption of vaccine strain recovered from the tablets to human cells is different depending on the cell lines used, HEK cells transformed by Ad 5 being the most efficient. This difference in the degree of adsorption to those human cell lines became noticeable probably due to partial or complete inactivation of penton fibers during the preparation of these tablets. Once these vaccine strains rescued from enteric coated tablets were passaged in 293 cells, the progeny virus particles with intact penton fibers could be titered in HeLa or KB cells.

### Plaque Assay

The original solution of Ad vaccine virus in 5 ml of MEM (containing an equivalent of the amount present in one tablet) supplemented with 5% gamma globulin (G.G) free calf serum and 10% glycerol was diluted ten fold for a plaque assay. Further serial dilutions were made from this ten-fold diluted stock. Aliquots of 0.5 ml were used for infecting cells 60-70% confluent in 60mm dishes. The adsorption of the virus was carried out for 45 min. at 37°C in the CO<sub>2</sub> incubator and the excess virus solution was removed at the end of incubation period. The cells were overlaid with a medium containing 0.5% agarose (SeaKem, Marine Colloids Div. FMC Corporation, P.O., Box 308, Rockland, ME 04841 USA), 10% G-G-free calf serum, 4mM glutamine in MEM containing standard amounts of penicillin, streptomycin and sodium bicarbonate without phenol red. The indicated concentration of DEAE-dextran and dimethyl sulfoxide (DMSO) were added in the first agarose overlay. Additional 5 ml of overlay medium was added every third or fourth day. When some CPE could be detected microscopically in the cell sheet, 5 ml of 0.5% agarose overlay medium containing neutral red (1 part in 20,000) was added. The plaques were counted for five days after adding the neutral red.

### Results

The results obtained in these studies are summarized as follows.

- a) Ad 4 virus from enteric coated vaccine tablets, diluted ten fold showed CPE on the fourth day in 293 cells. The number of plaques formed increased reaching a maximum on the tenth day and the size of the plaques reached a maximum on the 14th day (Fig. 8a & 8b). However, no CPE was observed over a period of 14 days in HeLa or KB cells.
- b) The effect of addition of varying concentrations of DEAE-dextran from 0.001% to 0.02% either alone or in the presence of 1% DMSO in the standard overlay medium was studied in plaque formation in 293, HeLa and KB cells. Fig. 8a shows the growth curves of Ad 4 vaccine strain under different concentrations of DEAE-dextran in the presence of constant amount of DMSO (1%). Increasing the concentration of DEAE-dextran hastens the CPE and plaque formation, reaching the maximum at 0.01%. On the 10th day, the number of plaques and their size morphology formed reached a maximum. Increasing the concentration of DEAE-dextran had an effect only on the size morphology but no effect on the total number.
- c) Either DMSO or DEAE-dextran alone did not show any enhancement of plaque formation (Fig. 8a & 9a). The growth curve in the presence of either DMSO or DEAE-dextran alone was similar to the one obtained in the absence of both DMSO and DEAE-dextran.
- d) In the presence of 0.02% DEAE-dextran, CPE appeared even on the second day and the plaques began to appear on the fourth day. However, DEAE-dextran at the concentration of 0.02% did have a lethal effect on the cells and it was difficult to distinguish the plaques from the dead cells.
- e) Ad 7 vaccine strain present in the enteric coated tablets showed some CPE in 293 cells only on the 14th day in the absence of any treatment (see above Fig. 9). The plaques formed were very small and the total titer remained at  $2 \times 10^3$  PFU per 5 ml of original virus suspension (the amount present per tablet) on the 16th day.
- f) The same conditions established for titrating Ad 4 vaccine strain were used to titer Ad 7 strain. As in the case of Ad 4, DMSO (1%) + DEAE-dextran treatment (0.01%) enhanced the formation of Ad 7 plaques in 293 cells. Plaques began to appear on the sixth day (2 days later than Ad 4 plaques under similar conditions) and increased in number reaching a plateau on the 14th day. Ad 7 plaques morphologically varied in size in contrast to those of Ad 4 which were more uniform in size. The titer of

the Ad 7 vaccine strain in the presence of DEAE-dextran (0.005%-0.01%) and DMSO (1%) reached a plateau at  $2 \times 10^4$ - $4.7 \times 10^4$  PFU/5ml (equivalent of one tablet).

Hence, the optimum system for plaque assay is the use of 293 cells as the host in the presence of 0.01% DEAE-dextran and 1% DMSO. It was reported by McCown et al. (1979) that a combination of DEAE-dextran and DMSO enhanced plaque formation of Phlebotomus fever virus in vero cells. No plaques were visible when DMSO was used alone or some plaques were barely visible when DEAE-dextran was used alone. In our experiments, 1% DMSO had no effect by itself in the enhancement of plaque formation of Ad 4 and Ad 7 vaccine strains. The presence of DEAE-dextran at a maximum allowable concentration of 0.01% had the maximum effect in the enhancement of plaque formation.

Ad 4 and Ad 7 vaccine tablets were assayed in 549 cells in the absence or presence of DEAE-dextran and DMSO and the results are shown in Fig. 10 and Fig. 11, respectively. Omission of DEAE-dextran and DMSO in the agar overlay reduced the size of the plaques (Fig. 10 or 11; 1 and 2) considerably. Although the addition of DEAE-dextran and DMSO enhanced the size and the number of the plaques formed, it did not have the same dramatic effect as in 293 cells (i.e.) have shortening the time required for the first appearance of plaques.

We studied the effect of addition of DEAE-dextran and DMSO on the efficiency of DNA transfection into 293 cells. Ad 7 DNA-protein complex was prepared from the virions as previously described (13, 14) and it was dialyzed against 10mM Tris-HCl (pH 7.4), 1mM EDTA, 0.1M NaCl and 1mM dithiothreitol. A monolayer of 293 cells (70% confluency) was used for DNA transfection. The cells were incubated with intact DNA-protein complex (Fig. 12a) or after digestion with *EcoRI* (Fig. 12b) (which is expected to abolish the infectivity of the DNA). Treatment of the *EcoRI*-digested DNA-protein complex with DNA ligase is expected to produce some viable DNA molecules (and hence plaques) (Fig. 12c). The efficiency of DNA transfection in the presence of DEAE-dextran and DMSO in the agar overlay seems to be better (Fig. 12a) compared to the one obtained by glycerol treatment generally used to enhance the DNA transfection (Fig. 12d).

#### Experiments to be Pursued or Completed During the Remainder of the Contract Year

- A. Experiments to be completed:
1. Physical mapping of Ad 4 (ATCC-VR4) strain and the vaccine strain.
  2. Physical mapping of Ad 21 vaccine strain and comparison with the prototype strain (ATCC).
  3. DNA sequence analysis at the Inverted Terminal Repetitions of Ad 4 and Ad 21. There seems to be a correlation between the length of inverted terminal repetition (ITR) of an Ad serotype and its oncogenicity in newborn hamsters. The ITR is shortest in non-oncogenic (Ad 2 & Ad 5) serotypes (103 base pairs) and longest in highly oncogenic (Ad 12 & Ad 18) Ads. The length of ITR for the weakly oncogenic group B (Ad 3, Ad 7) falls in the middle (136 base pairs). ITR of Ad 21 (a group B Ad) is unknown. Ad 4 has been reported to be non-oncogenic but it is classified into a unique group E from the DNA - DNA homology data. We will determine the length of ITR of Ad 4 and Ad 21 by actual sequence analysis.



Table 1. Restriction fragments of Ad 7 (Greider) strain

Fragment	<u>EcoRI</u>	<u>Sall</u>	<u>HpaI</u>	<u>SmaI</u>	<u>BamHI</u>	<u>KpnI</u>	<u>BclI</u>	<u>BstEII</u>	<u>HindIII</u>	<u>XbaI</u>
A	86.0 <sup>l</sup>	48.8	69.3 <sup>l</sup>	20.9	26.7	39.3 <sup>l</sup>	31.9 <sup>l</sup>	29.2 <sup>l</sup>	20.2	38.0
B	14.0 <sup>r</sup>	33.7 <sup>r</sup>	20.7	19.1	21.6	31.9 <sup>r</sup>	19.0	24.7	16.5 <sup>r</sup>	28.2 <sup>l</sup>
C		17.5 <sup>l</sup>	10.0 <sup>r</sup>	14.5	13.2	26.0	17.0	12.5	13.5	20.0 <sup>r</sup>
D				13.3 <sup>l</sup>	10.6	2.8	9.4 <sup>r</sup>	11.6	12.8	13.8
E				10.8	10.2		8.5	7.9	9.6	
F				6.7	7.6 <sup>r</sup>		7.9	6.6	9.3	
G				6.6	3.2		3.3	5.4	6.0	
H				5.6	2.7		3.0	2.2 <sup>r</sup>	4.6	
I				2.5 <sup>r</sup>	2.2				3.8 <sup>l</sup>	
J					2.0 <sup>l</sup>				3.7	

l, r = left and right terminal fragments, respectively.

Table II. Analysis of partial hydrolysis of Ad 7 digested with BclI

Parent DNA fragment	partial <u>BclI</u> cleavage products	Genome length (%)	Order of <u>BclI</u> fragments deduced
		<u>Estimated Size</u>	<u>Size difference</u>
<u>EcoRI-A</u>	a	86.0	<u>EcoRI-A</u> uncleaved by <u>BclI</u>
	b	51.6	34.4 F <sub>2</sub> -H-B-G-C-E
	c	45.5	6.1 F <sub>2</sub> -H-B-G-C
	d	29.8	15.7 F <sub>2</sub> -H-B-G
	e	26.9	2.9 F <sub>2</sub> -H-B
	f	5.2	21.7 F <sub>2</sub> -H
	g	2.4	2.8 F <sub>2</sub>
<u>EcoRI-B</u>	a	12.8	<u>EcoRI-B</u> uncleaved by <u>BclI</u>
	b	3.1	9.7 F <sub>1</sub>
Whole Ad 7 labeled at 5'-termini with [ <sup>125</sup> I]	a	60.3	A-E-C-G
	b	56.3	4.0 A-E-C
	c	40.2	16.1 A-E
	d	31.9	8.3 A
	e	19.3	D-F-H
	f	16.1	3.2 D-F
	g	9.4	6.7 D

Table II continued.

<u>EcoRI</u> A + B mixture labeled with [ <sup>32</sup> P] at the 5' terminus of <u>EcoRI</u> site	a	86.0		uncleaved <u>EcoRI</u> -A
	b	51.6		F <sub>2</sub> -H-B-G-C-E
	c	45.5	6.1	F <sub>2</sub> -H-B-G-C
	d	29.8	15.7	F <sub>2</sub> -H-B-G
	e	26.9	2.9	F <sub>2</sub> -H-B
	f	12.8	14.1	F <sub>1</sub> -D ( <u>EcoRI</u> -B)
			7.6	
	g	5.2		F <sub>2</sub> -H

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lower case letters refer to partial digest products of BclI upper case letters refer to fragments produced with complete digestion with BclI (in this case, they represent terminal fragment.

Table III. ENDONUCLEASE R FRAGMENTS PRODUCED BY DOUBLE DIGESTION

Endonuclease Pairs								
<u>Bam</u> HI + <u>Bcl</u> II	<u>Bcl</u> II + <u>Kpn</u> I	<u>Sma</u> I + <u>Hpa</u> I	<u>Bcl</u> II + <u>Hpa</u> I	<u>Sal</u> I + <u>Hind</u> III	<u>Xba</u> I + <u>Kpn</u> I	<u>Bst</u> EII + <u>Kpn</u> I	<u>Bam</u> HI + <u>Sal</u> I	<u>Bst</u> EII + <u>Bam</u> HI
21.9	31.9	20.2	31.9	20.2	28.2	29.2	23.6	19.1
17.0	15.0	14.5	17.0	16.5	26.0	24.7	18.6	15.8
11.1	11.8	13.3	12.0	13.5	20.0	12.5	13.3	10.0
7.9	9.4	12.0	9.4	12.0	11.0	11.6	10.5	8.6
7.5	7.9	8.8	8.5	9.6	2.5		8.6	6.6
7.5	7.4	6.7	7.9	9.3			7.8	6.3
4.4	7.4	6.7	6.7	4.6		6.5	7.8	6.2
3.8	3.3	6.6	3.3	3.8		5.2	3.2	5.4
3.2	3.0	5.6	3.0	3.7		5.0	2.5	5.2
2.7	1.7	2.7		3.6		2.8	2.2	4.4
2.5	1.1	1.7		2.7		2.5	2.1	3.2
2.3							2.0	2.7
2.2								2.3
2.0								2.2
1.8								2.0
1.0								1.6

Table IV. Analysis of partial hydrolysis products of Ad 7 digested with BamHI

Parent DNA fragment	partial <u>Bam</u> HI cleavage products	Genome length (%)	Order of <u>Bam</u> HI fragments deduced
		<u>Estimated Size</u>	<u>Size difference</u>
<u>Eco</u> RI-A (5'- [ <sup>125</sup> I]labeled)	a	38.6	J-G-H-I-A
	b	10.2	28.4 J-G-H-I
	c	7.9	2.3 J-G-H
	d	5.3	2.6 J-G
	e	1.9	3.4 J
<u>Eco</u> RI-B (5'- [ <sup>125</sup> I]labeled)	a	12.4	D <sub>1</sub> -F ( <u>Eco</u> RI-B uncleaved)
	b	7.4	5.0 F
<u>Eco</u> RI-A (labeled with [ <sup>32</sup> P] at <u>Eco</u> RI site)	a	86.0	D <sub>2</sub> -C-E-B-A ( <u>Eco</u> RI-A)
	b	54.9	31.1 D <sub>2</sub> -C-E-B
	c	30.4	24.5 D <sub>2</sub> -C-E
	d	18.6	11.8 D <sub>2</sub> -C
	e	4.0	14.6 D <sub>2</sub>
<u>Eco</u> RI-B (labeled with [ <sup>32</sup> P] at <u>Eco</u> RI site)	a	14.3	D <sub>1</sub> -F ( <u>Eco</u> RI-B)
	b	6.2	8.1 D <sub>1</sub>

Table V. Analysis of partial hydrolysis products of Ad 7 digested with BstEII and SmaI

Enzyme used	Parent DNA fragment	partial <u>BstEII</u> cleavage products	Genome length (%)	Order of <u>BclI</u> fragments deduced
			<u>Estimated Size</u>	<u>Size difference</u>
<u>BstEII</u>	[ <sup>125</sup> I]- <u>EcoRI</u>	a	67.9	A-F-E-R
		b	43.9	A-F-E
		c	37.1	A-F
		d	31.2	A
<u>BstEII</u>	whole Ad 7 labeled at 5'-termini with [ <sup>125</sup> I]	a	56.7	H-D-C-G-R
		b	31.2	H-D-C-G or A
		c	25.2	H-D-C
		d	13.6	H-D
		e	2.5	H
<u>SmaI</u>	[ <sup>125</sup> I]- <u>EcoRI</u>	a	60.9	D-H-G-A-C
		b	44.8	D-H-G-A
		c	24.9	D-H-G
		d	18.8	D-H
		e	13.3	D

### Figure Legends

- Fig. 1. Physical mapping of terminal fragments of restriction endonucleases: BamHI (a & b), KpnI (c & D), SalI (e & f), HpaI (g & h), EcoRI (i & j), BclI (k & l), SmaI (m & n), XbaI (o & p), XhoI (q & r), HindIII (s & t) and BstEII (u & v). a, c, e, g, i, k, m, o, q, s and u represent ethidium bromide fluorescence of DNA bands. b, d, f, h, j, l, n, p, r, t and v represent the autoradiography of the above gels.
- Fig. 2. Physical mapping of cleavage sites of restriction endonucleases by the method of partial hydrolysis (Smith and Birnsteil, 1976). Autoradiography of the gel containing the products of:  
a. Partial hydrolysis of [<sup>125</sup>I]-EcoRI-B with SmaI  
b. Partial hydrolysis of [<sup>125</sup>I]-EcoRI-A with SmaI  
c. Partial hydrolysis of [<sup>125</sup>I]-EcoRI-B with BamHI  
d. Partial hydrolysis of [<sup>125</sup>I]-EcoRI-A with BamHI  
e. & f. longer-exposure of the gel d & c to Xray film to bring out the weak bands.
- Fig. 3. Physical mapping of cleavage sites of restriction endonucleases by the method of partial hydrolysis.  
Auto-radiography of the gel containing the products of:  
a. & b. partial hydrolysis of [<sup>125</sup>I]-EcoRI-A with BstEII  
c. & d. partial hydrolysis of [<sup>125</sup>I]-Ad 7 DNA with BclI  
The two lanes in the middle represent the complete hydrolysis of [<sup>125</sup>I]-Ad 7 DNA with BstEII. The terminally labeled A and H fragments are seen.
- Fig. 4. Physical mapping of cleavage sites of restriction endonucleases by the method of partial hydrolysis.  
Auto-radiography of the gel containing the products of:  
a. & b. partial hydrolysis of [<sup>125</sup>I]-Ad 7 with BstEII  
c. & d. partial hydrolysis of [<sup>125</sup>I]-Ad 7 with BamHI
- Fig. 5. Map of the restriction endonuclease cleavage sites on Ad 7 DNA.
- Fig. 6. Comparison of the restriction enzyme cleavage patterns of Ad 7 (Greider) and Ad 7 vaccine strain.  
Lanes 1, 3, 5, 7, 9, 11, 13, 15, and 17 contained Ad 7 Greider DNA and the even numbered lanes contained the Ad 7 DNA from vaccine strain. Lanes 1 & 2, undigested DNA; 3 & 4, EcoRI; 4 & 5, SalI; 6 & 7, XbaI; 8 & 9, BamHI; 10 & 11, BclI; 12 & 13, BstEII; 14 & 15, HindIII; 16 & 17, SmaI.
- Fig. 7. Restriction endonuclease cleavage patterns of Ad 4 (ATCC, VR-4).  
Lanes represents as follows: 1 (BclI); 2(HindIII); 3(SalI); 4 (EcoRI); 5(XbaI); 6(BamHI); 7(BstEII); 8(SmaI); 9( $\lambda$  DNA digested with EcoRI + HindIII served as molecular weight markers).

Fig. 8. Titration of Ad 4 vaccine strain from enteric coated tablets in 293 cells. DEAE-dextran or dimethyl sulfoxide (DMSO) were used in the agar overlay medium.

a. ●—●, no addition of DMSO or DEAE-dextran; x—x, DMSO (1%) only; Δ—Δ, 0.01% DEAE-dextran only; ⊗—⊗, 0.001% DEAE-dextran + 1% DMSO; □—□, 0.005% DEAE-dextran + 1% DMSO; ○—○, 0.01% DEAE-dextran + 1% DMSO.

b. An experiment in which 1) neither DMSO or DEAE-dextran were included, 2) only DMSO (1%) was added and 3) both DMSO (1%) and DEAE-dextran (0.01%) were added, was carried out. The plaques were photographed after staining with neutral red on the 14th day. The plaques in 3 were morphologically similar to 2 at the end of 12th day. However, the cytotoxic effect of DEAE-dextran was beginning to appear at the end of 14th day. The number of plaques reached a plateau after 10th day (Fig. 7a).

Fig. 9. Titration of Ad 7 vaccine strain from enteric coated tablets in 293 cells.

a. ●—●, no addition of DMSO or DEAE-dextran; x—x, DMSO (1%) only; Δ—Δ, 0.01% of DEAE-dextran only; ⊗—⊗, 0.005% of DEAE-dextran + DMSO (1%); □—□, 0.007% of DEAE-dextran + DMSO (1%); ○—○, 0.01% of DEAE-dextran + DMSO (1%).

b. 1. no addition of DEAE-dextran or DMSO 2. 0.001% DEAE-dextran + 1% DMSO 3. 0.005% of DEAE-dextran + 1% DMSO. 4. 0.01% of DEAE-dextran + 1% DMSO. The virus suspension from Ad 7 vaccine tablets was diluted 10 fold.

c. The virus suspension was diluted 100 fold. 1. no addition of DEAE-dextran or DMSO. 2. 0.005% of DEAE-dextran + 1% DMSO. 3. 0.01% of DEAE-dextran + 1% DMSO.

Fig. 10. Titration of Ad 4 vaccine strain from enteric coated tablets assayed in 549 cells. 1 and 2. DEAE-dextran and DMSO were omitted in the agar overlay medium. The virus suspension was diluted 10 fold in 1 and 3 and 100 fold in 2 and 4.

Fig. 11. Titration of Ad 7 vaccine strain from enteric coated tablets assayed in 549 cells. 1-4 represent the same as described in Fig. 10 except Ad 7 strain was used.

Fig. 12. Effect of addition of DEAE-dextran and DMSO to the efficiency DNA transfection. 293 cells were incubated with Ad 7 DNA-protein complex according to the method described by Graham and van der Eb. After incubation for 2 hours at 37°C, the cells were overlaid with 5ml of agar containing MEM, antibiotics and DEAE-dextran (0.01%) and DMSO (1%). On the third and sixth day, additional agar overlay medium (3ml each) was added. The cells were stained with neutral red after 12 days & photographed.

a. Intact Ad 7 DNA-protein complex

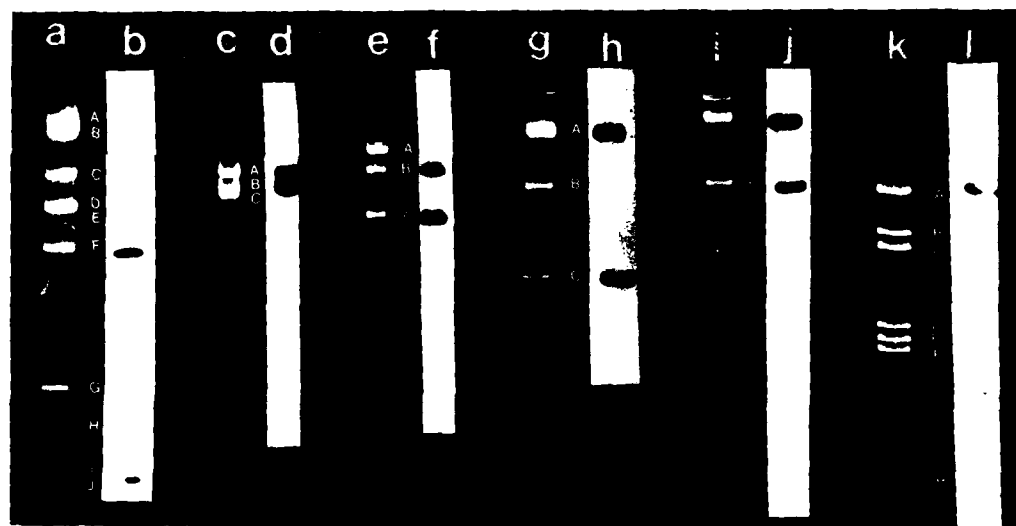
b. DNA-protein complex digested with *EcoI*.

c. *EcoRI* digested DNA-protein complex treated with DNA ligase.

d-f. The DNA samples were the same as in a-c, except that treatment with glycerol (subsequent to the incubation of cells with DNA-protein complex, treatment with 15% glycerol for 30 seconds, followed by agar overlay) was carried out.



FIGURE 1



**FIGURE 2**

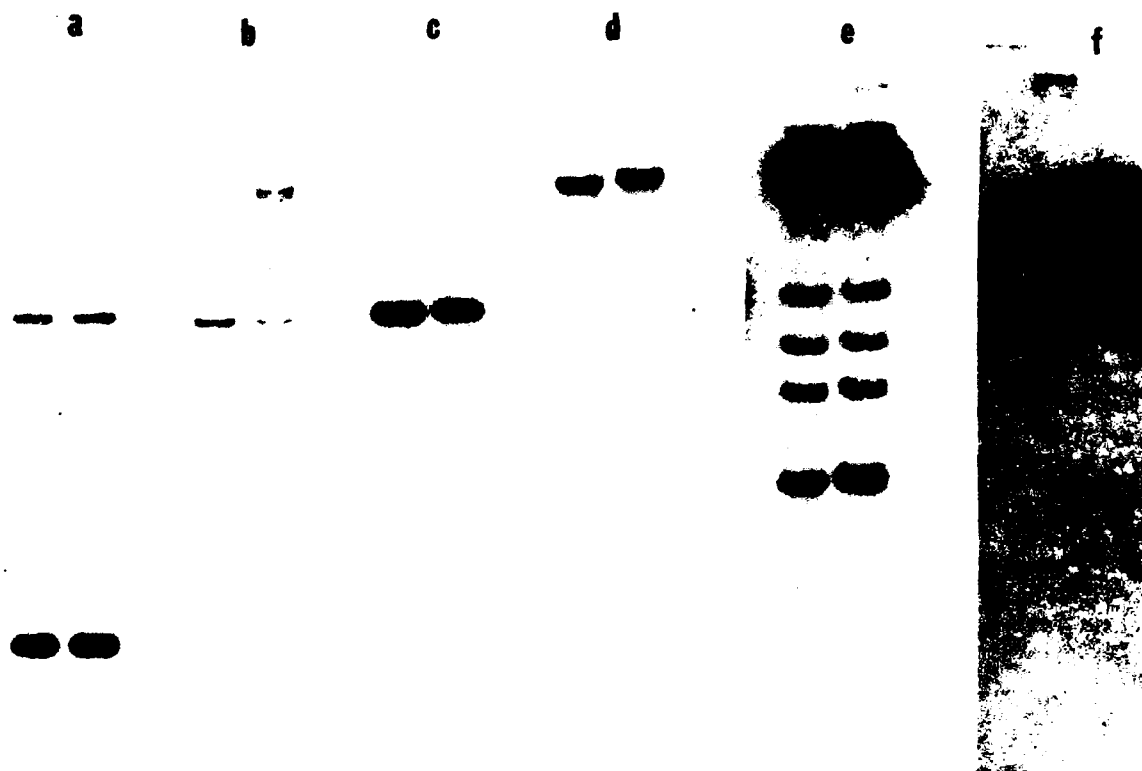


FIGURE 3

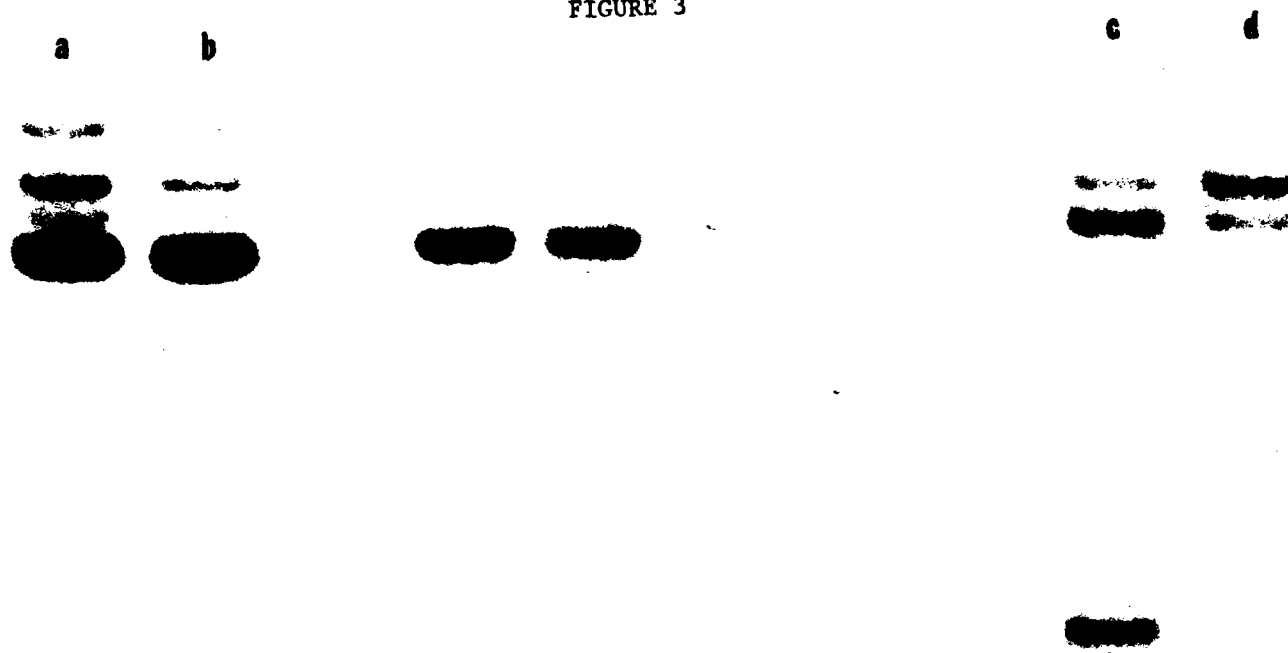


FIGURE 4

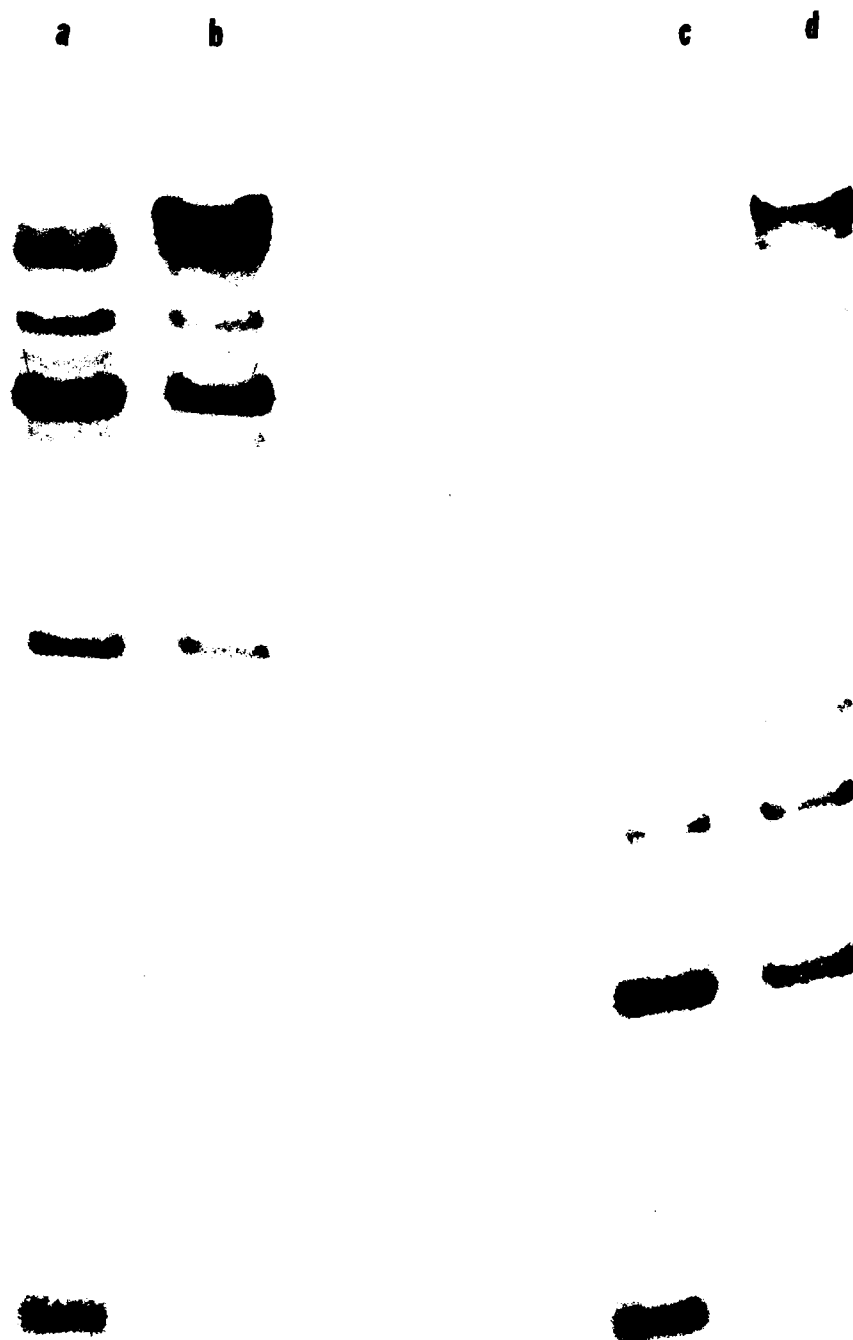
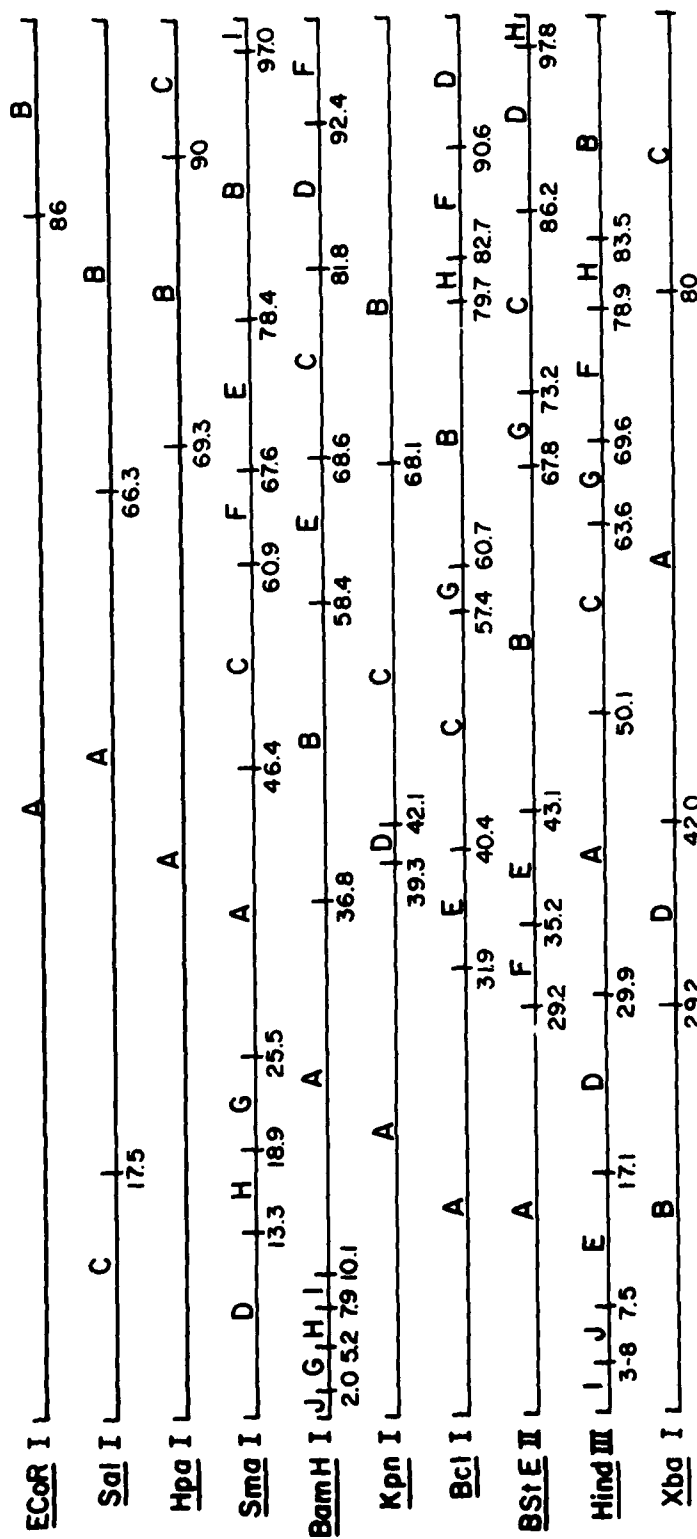


FIGURE 5



MAP UNITS

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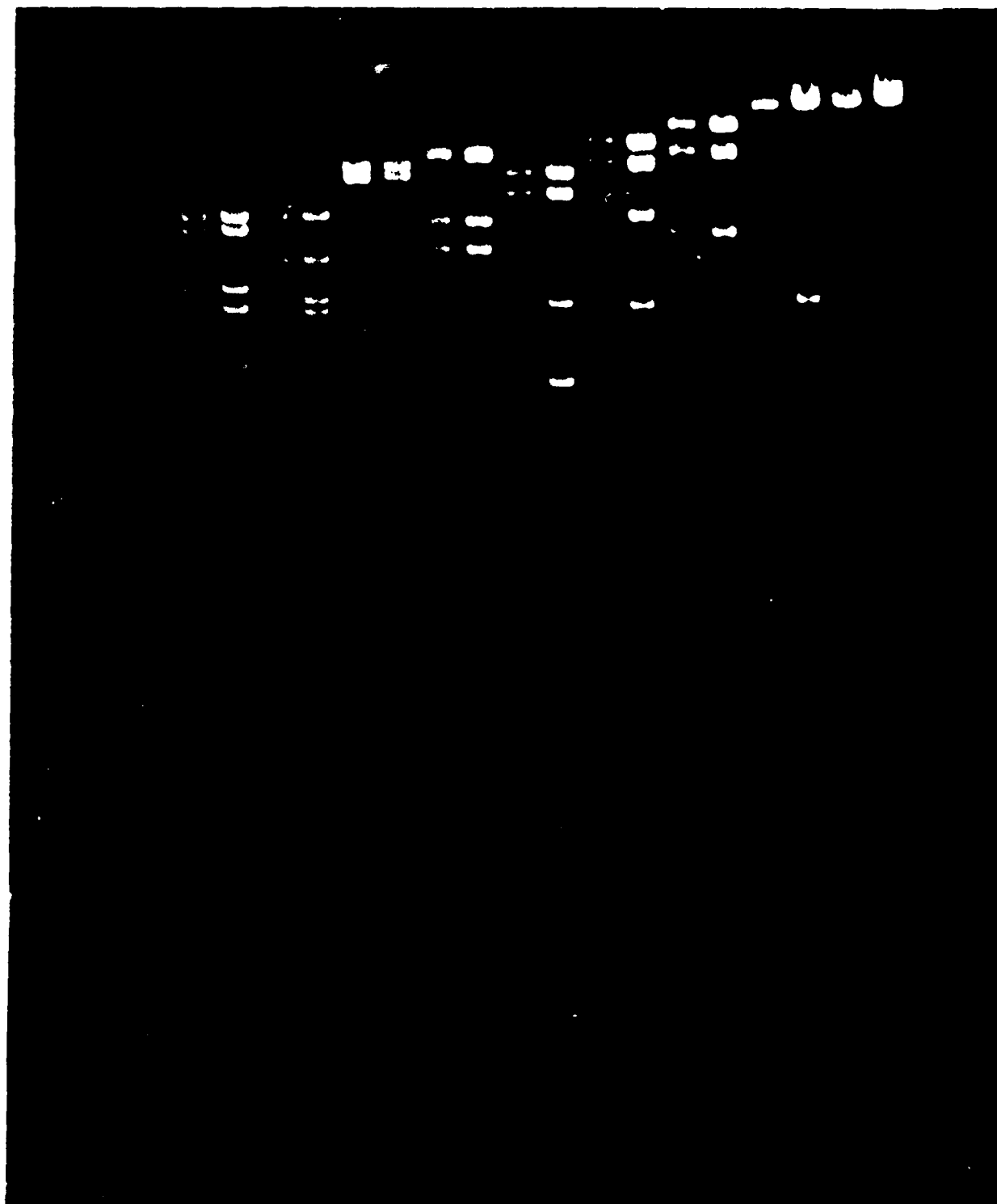


FIGURE 6

9 8 7 6 5 4 3 2 1

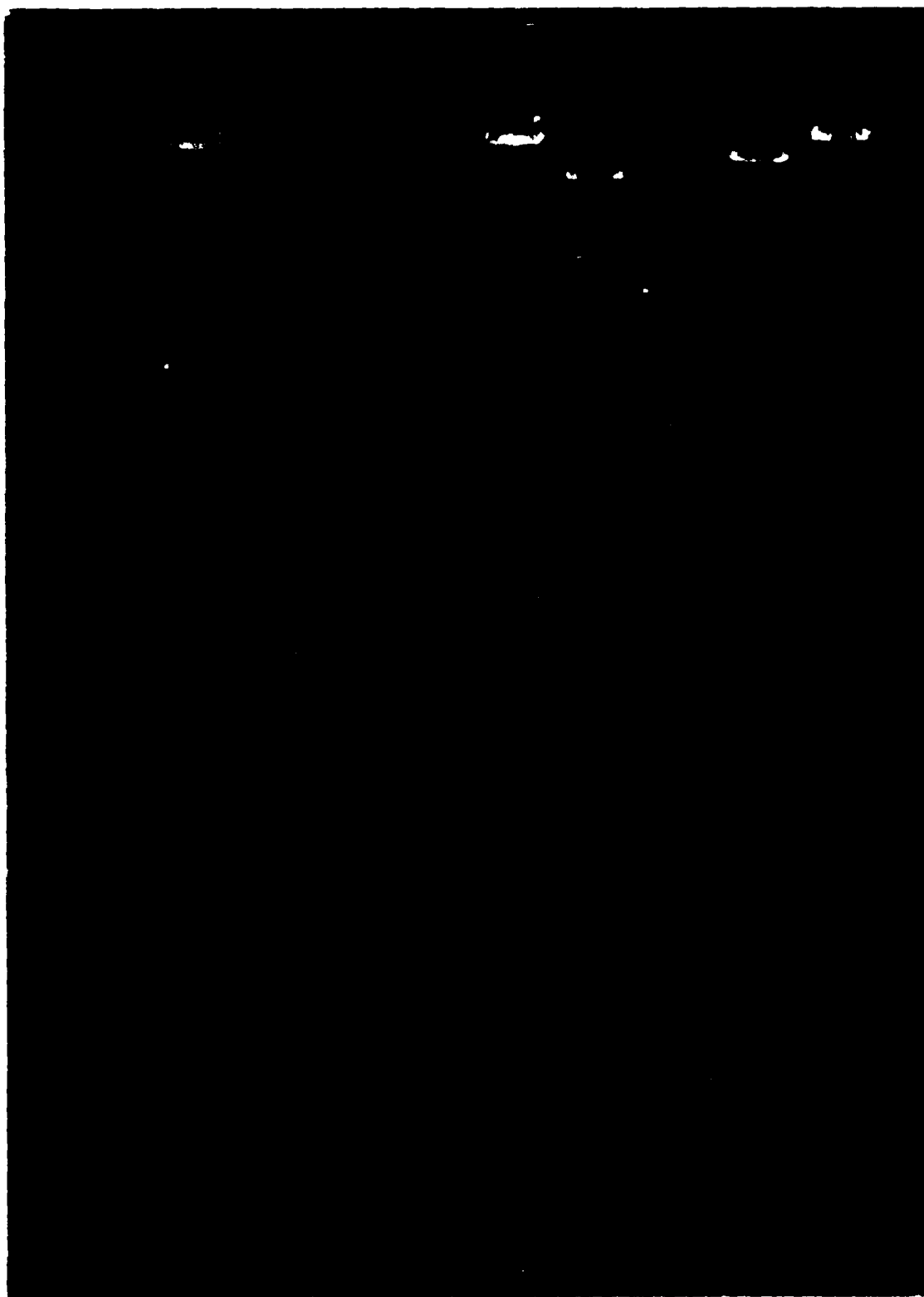
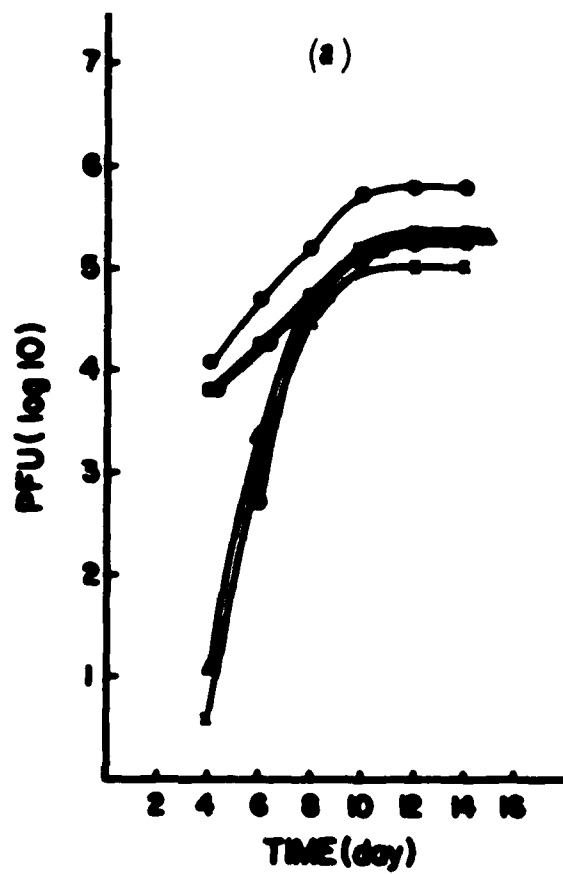
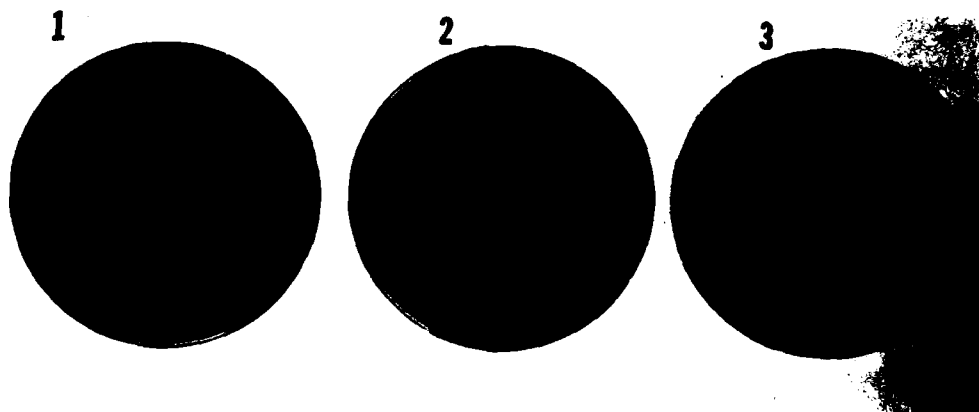


FIGURE 7

FIGURE 8



(b)





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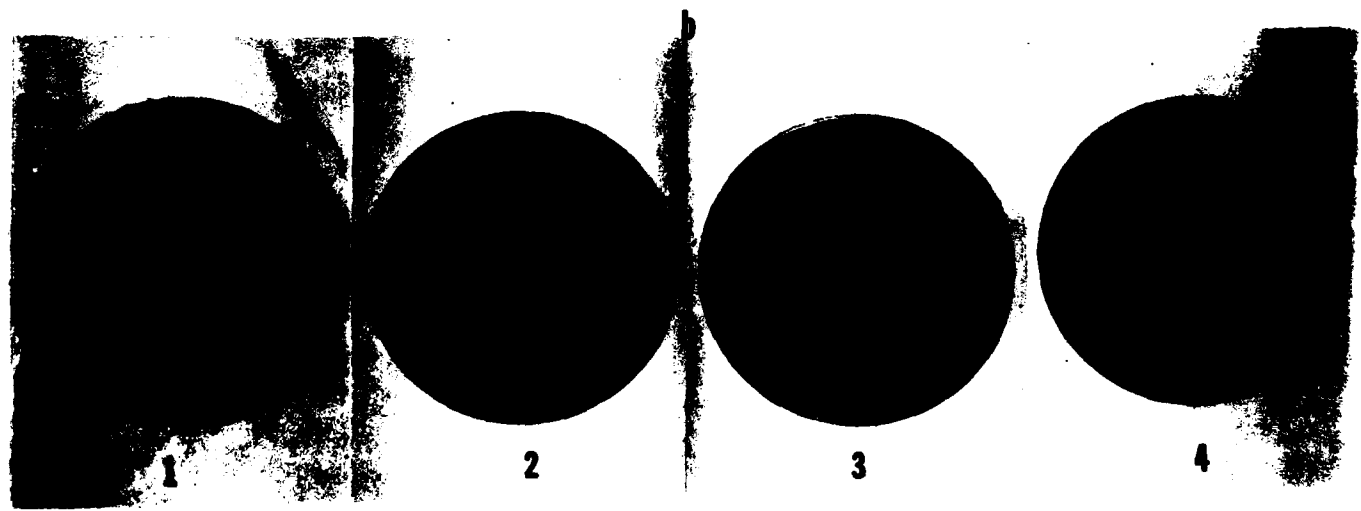
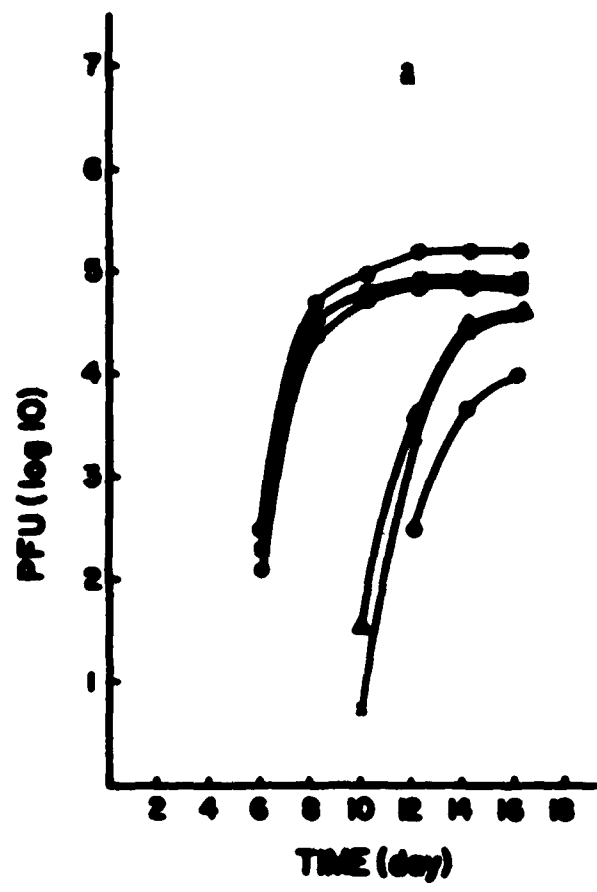


FIGURE 10

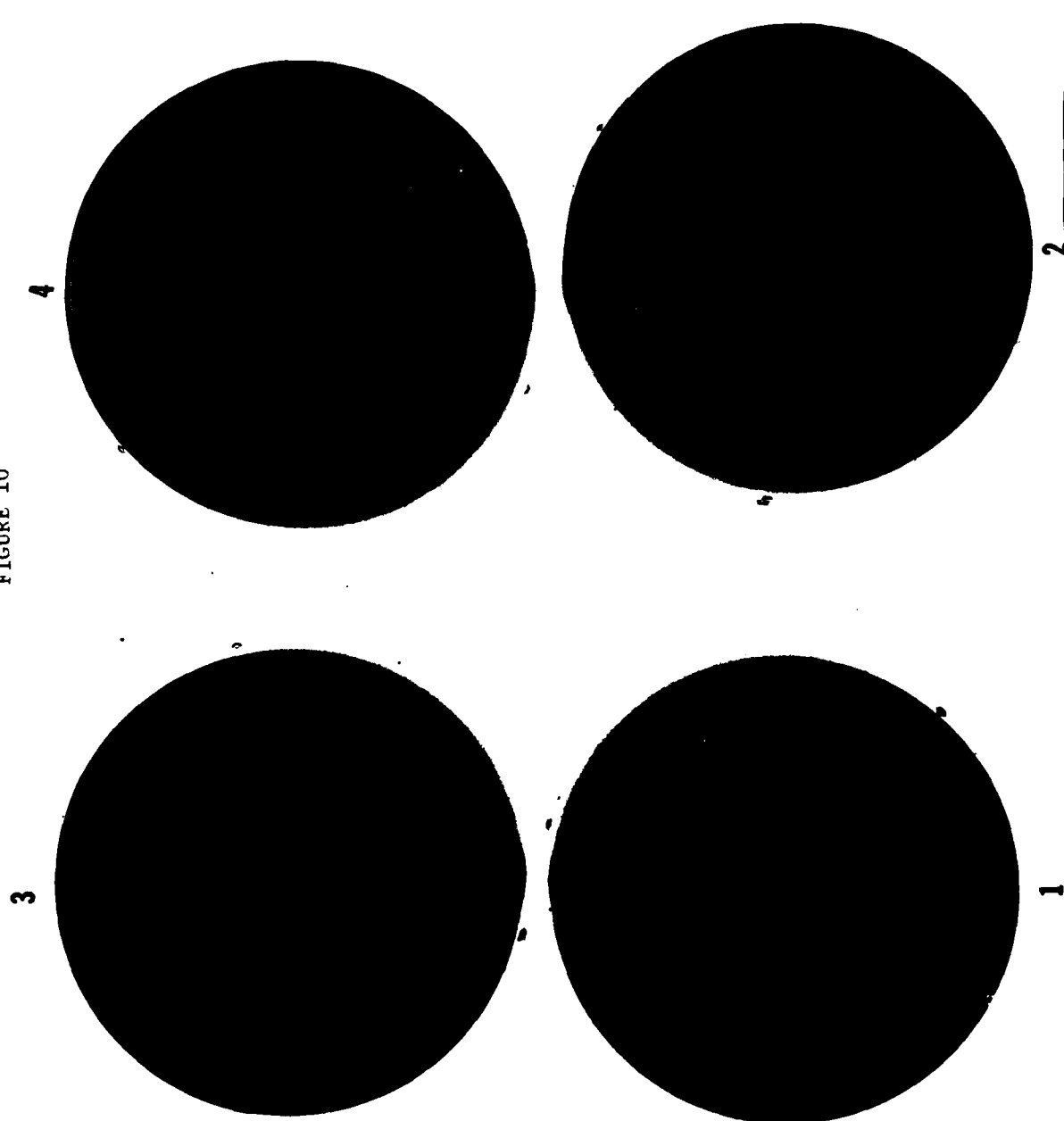
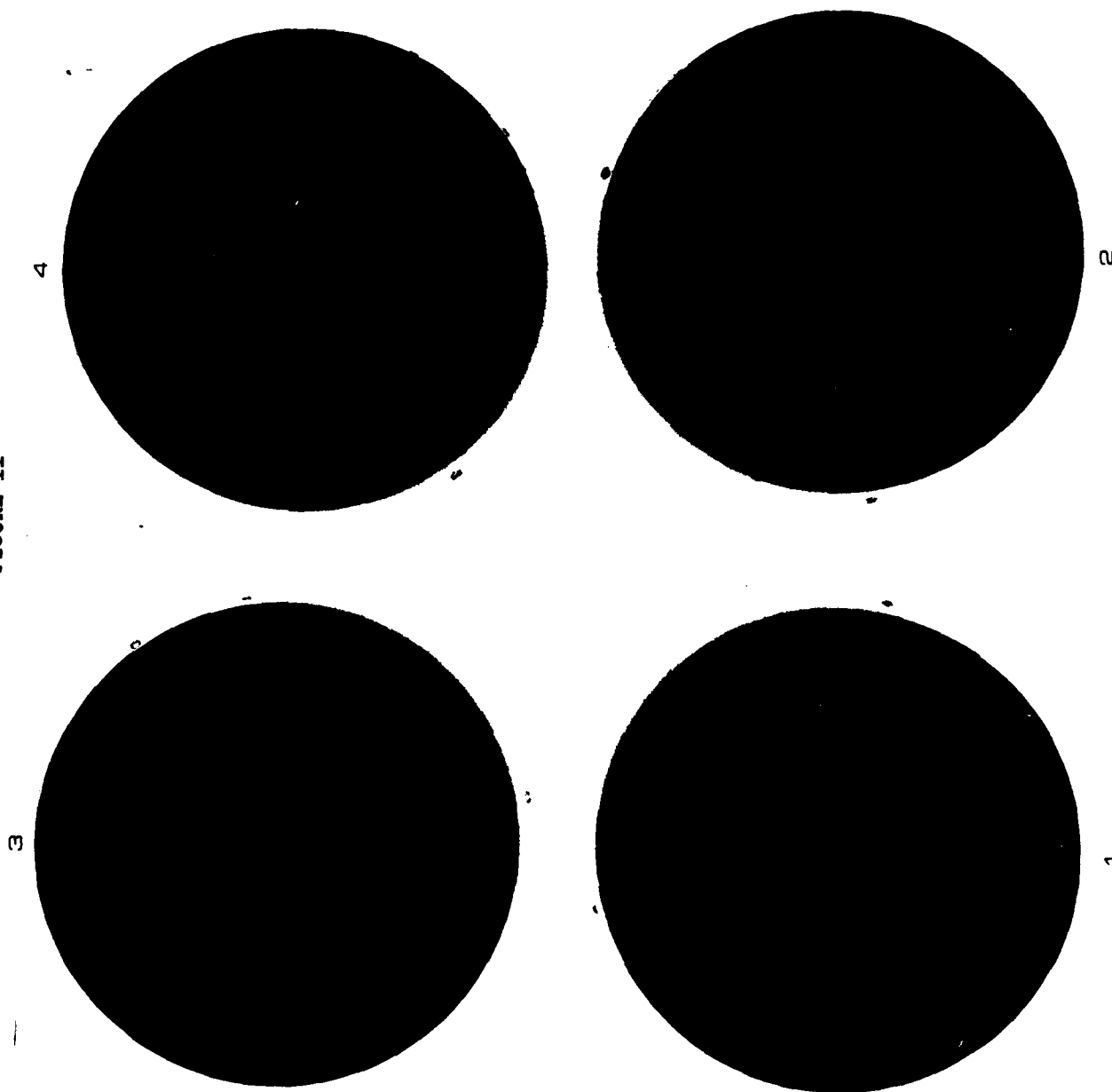
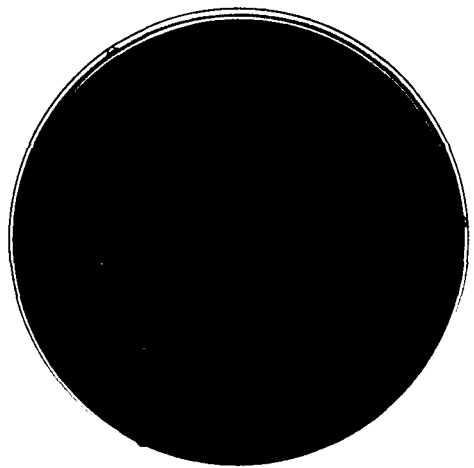
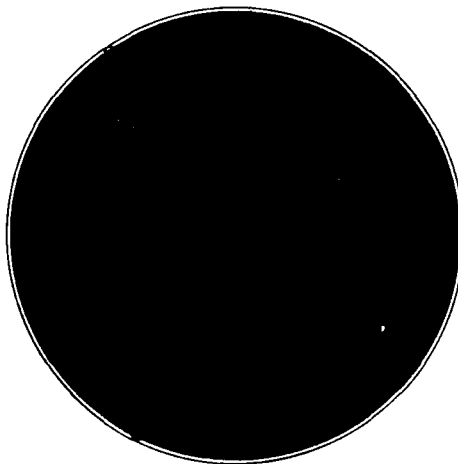


FIGURE 11

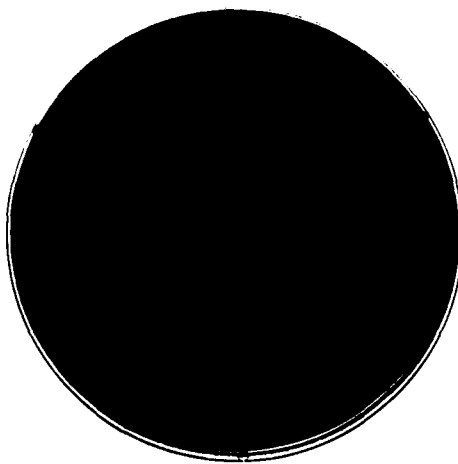




c



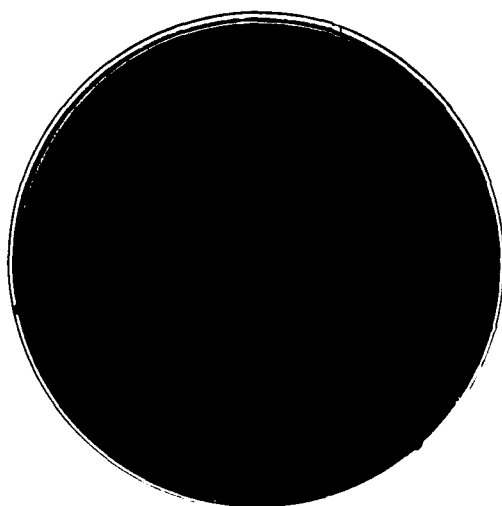
b



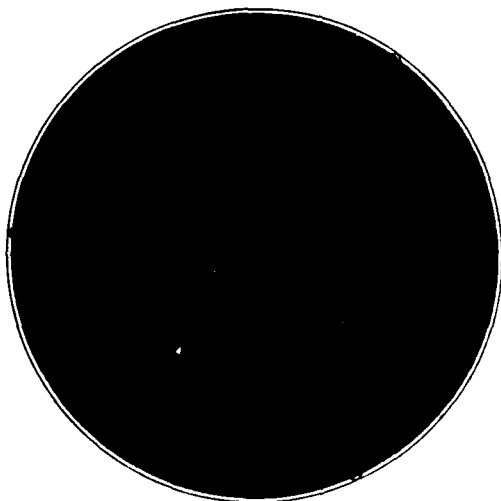
a

FIGURE 12

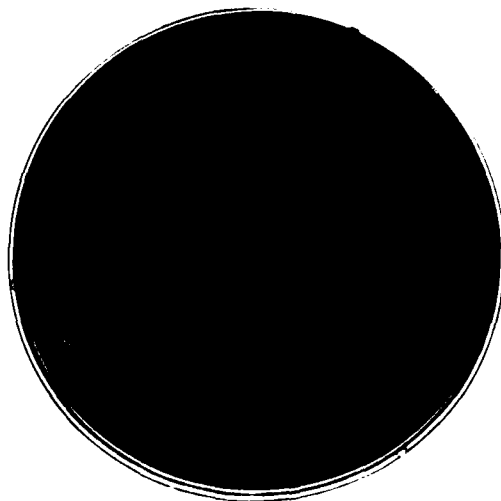
FIGURE 12



f

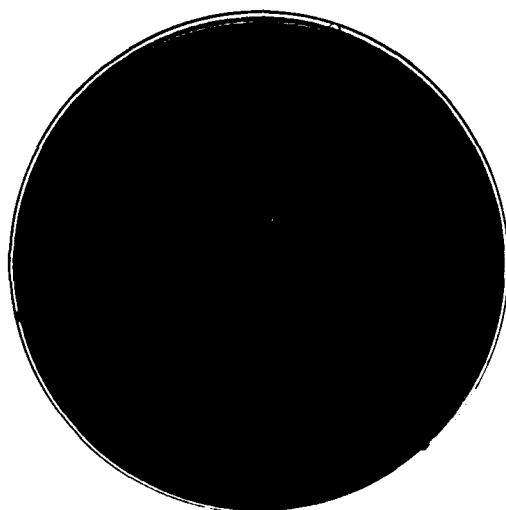


e

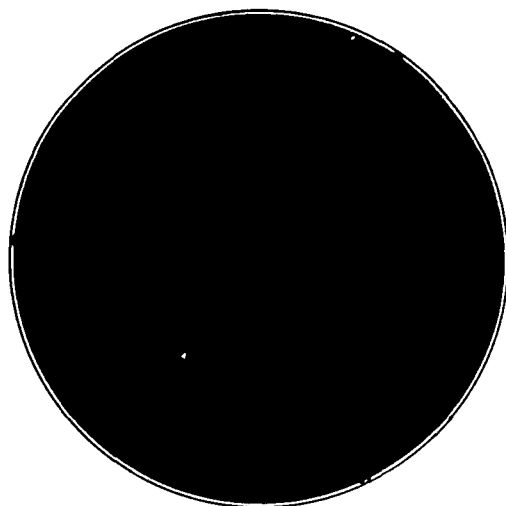


d

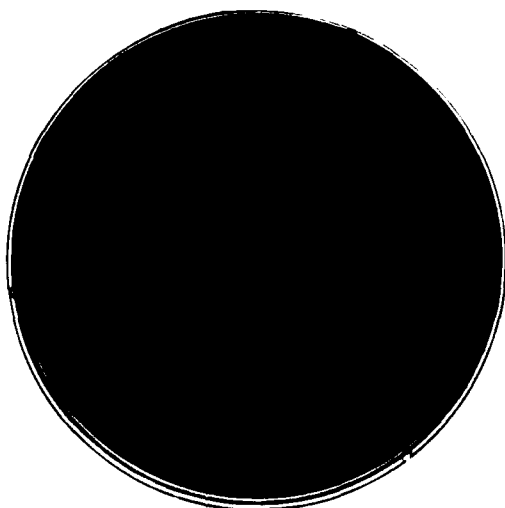
FIGURE 12



f



e



d

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STUDIES ON THE NATURE OF THE LINKAGE BETWEEN THE TERMINAL PROTEIN  
AND THE ADENOVIRUS DNA

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**Summary:** The termini of human adenovirus types 7 and 2 DNA extracted from the virions using pronase and protease K are covalently linked to peptides that contain tyrosine residue(s). The peptide attached to each terminus can be labeled with [<sup>125</sup>I] in vitro. The linkage between the peptide moiety and the terminal nucleotide is sensitive to snake venom phosphodiesterase, *Aspergillus* nuclease S<sub>1</sub> and micrococcal nuclease. The available data suggest that the peptide is linked to the terminal nucleotide of these DNA molecules through a phosphodiester bond.

Human Ad\* DNA extracted from the virion using guanidine hydrochloride is a linear, duplex molecule containing a protein of 55,000 daltons tightly associated with its termini (1-8). Treatment of the Ad virion or the DNA-protein complex with a protease results in a linear DNA whose 5' ends were blocked to digestion with 5' exonucleases and phosphorylation by phosphatase, polynucleotide kinase and [ $\gamma$ -<sup>32</sup>P] ATP (9). However, the 3'-specific *E. coli* exonuclease III acts on such viral DNA molecules (10,11,12). These observations suggested that the 55K protein bound (presumably covalently) to the 5' termini leaves a residual amino acid or peptide attached to the 5' terminal nucleotide upon treatment with protease K or pronase. The 5' terminal nucleotide has been shown to be a dC residue (9,13-15). In this communication, we wish to report that the viral DNA isolated from Ad 2 or Ad 7 serotype by treatment with protease K and/or pronase can be specifically labeled at the 5' termini with [<sup>125</sup>I] using chloramine-T as the oxidizing agent (16). Although under some circumstances iodine may react with sulf-

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\* Abbreviations used: Ad, Adenovirus



hydroxyl groups or with tryptophan, the chemistry of iodination is essentially the chemistry of substitution of iodine into tyrosine groups (17). We also present evidence that a peptide containing tyrosine residue is most likely bound to the 5' phosphate of DNA through a phosphodiester bond.

**MATERIALS AND METHODS:** Ad2 and Grider Strain of Ad7 were grown in KB cells, and virions purified as described (18). The dialyzed virus suspension (10 ml) was treated with pronase (100  $\mu$ g/ml; Calbiochem) in an incubation mixture containing 0.2 mM mercaptoethanol, 10 mM Tris-HCl, pH 7.2 and 5 mM EDTA for 30 min at 37°C. Subsequently the reaction mixture was treated with 500  $\mu$ g of protease K (EM Laboratories) in the presence of 0.1% SDS at 37°C for 30 min. The DNA was phenol extracted and dialyzed against 0.01 M Tris-HCl, pH 7.4 containing 1 mM EDTA and precipitated with ethanol before labeling with [<sup>125</sup>I]. Iodination was carried out in the absence of SDS.

**[<sup>125</sup>I] labeling of Ad DNA:** Ad7 DNA (78  $\mu$ g) or Ad2 DNA (60  $\mu$ g) was dissolved in 100  $\mu$ l of 0.05 M sodium phosphate, pH 7.5 in an Eppendorf tube (1.5 ml capacity). Chloramine-T (2  $\mu$ l of 1 mg/ml) and 1 mCi of carrier-free Na [<sup>125</sup>I] (Amersham Corp.) were then added and the contents of the tube were mixed well. The reaction mixture was incubated in ice for 30 min. The reaction was stopped by the addition of 5  $\mu$ l of sodium metabisulfite (1 mg/ml) and 10  $\mu$ l of KI (10 mg/ml) and the mixture was diluted to 300  $\mu$ l with H<sub>2</sub>O. A 50  $\mu$ l aliquot of 0.02% bromophenol blue and 50% glycerol was added and the mixture was applied to an agarose column (1.5 M from BioRad Corp.) packed in a plastic disposable pipet of 10 ml capacity. The column was eluted with 0.01 M Tris-HCl, pH 7.5, 0.001 M EDTA and 0.1 M NaCl. The [<sup>125</sup>I] labeled DNA, was precipitated with two volumes of ethanol. The precipitate was dissolved in 100  $\mu$ l of 0.01 M Tris-HCl, pH 7.4, containing 1 mM EDTA.

**Enzymatic Digestion:** Ad7 DNA was digested with pancreatic DNase I in an incubation mixture (50  $\mu$ l) containing 7.8  $\mu$ g of DNA, 6 mM MgCl<sub>2</sub>, 60  $\mu$ g of DNase I and 1 mM phenyl methyl sulfonyl fluoride. Incubation was carried out at 37°C for 60 min. At the end of the reaction, 25  $\mu$ l of the reaction mixture was treated at 37°C for 2 h with 37.5  $\mu$ g of snake venom phosphodiesterase (Worthington Biochemical Corp.) in an incubation mixture containing 50 mM Tris-HCl, pH 8.0 and 10 mM MgCl<sub>2</sub>. An aliquot of [<sup>125</sup>I] labeled Ad7 DNA (7.8  $\mu$ g) was denatured by heating in a boiling water bath for 5 min, and quickly cooled in ice. It was subsequently treated with single-strand specific S<sub>1</sub> nuclease (20 units) at 37°C for 1 h in an incubation mixture (20  $\mu$ l) containing 2.5  $\mu$ g of denatured calf thymus DNA, 5 mM ZnSO<sub>4</sub>, 30 mM sodium acetate, pH 4.6 and 5% glycerol.

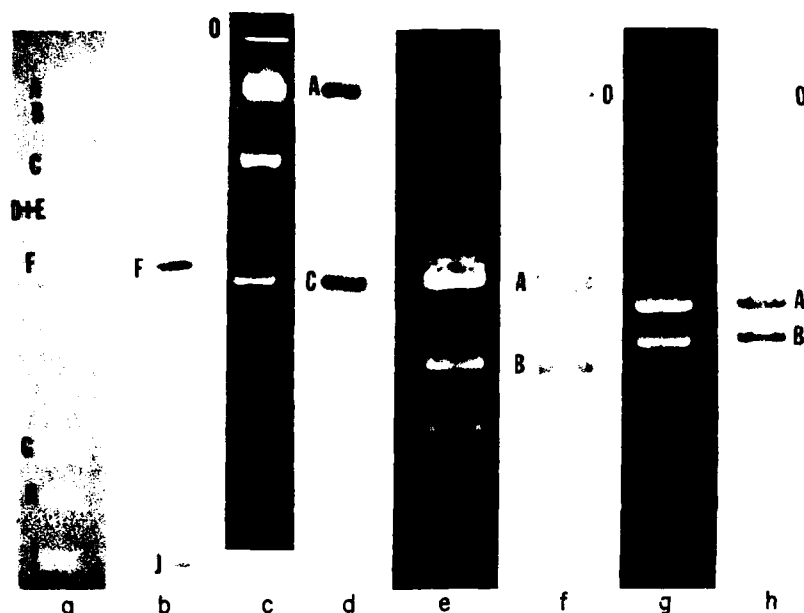
**Staphylococcus aureus (micrococcal) nuclease digestion of [<sup>125</sup>I] labeled Ad7 DNA** (2  $\mu$ g) was carried out in an incubation mixture (50  $\mu$ l) containing 10  $\mu$ g of calf thymus DNA, 20 mM Tris-HCl, pH 8.0, 5 mM CaCl<sub>2</sub> and 300 units of the enzyme at 37°C for 1 h.

**[<sup>125</sup>I] labeled Ad7 DNA-protein complex:** The DNA terminal protein complex of Ad7 was purified by the method described by Robinson et al. (1) and labeled with [<sup>125</sup>I] as described above. The labeled complex was dialyzed to remove unbound [<sup>125</sup>I], digested with protease K, extracted with phenol and precipitated with ethanol.

**Acid hydrolysis of [<sup>125</sup>I] labeled Ad DNA:** The labeled DNA was hydrolyzed in sealed glass ampules under nitrogen in the presence of 20  $\mu$ g of bovine serum albumin carrier protein in 200  $\mu$ l of 2 M HCl at 110°C for 24 h.

**3MM Paper Ionophoresis:** The enzyme digests were spotted on a 34 cm length of Whatman No. 3MM paper and subjected to electrophoresis in pH 1.9 formic/acetic acid buffer system as described (19).

**RESULTS AND DISCUSSION:** The [<sup>125</sup>I] labeled Ad DNA was analyzed by cleaving with restriction enzymes. Fig. 1a,c,e and g show the ethidium bromide fluorescence (20) and fig. 1b,d,f and h show the autoradiography of 1.4% agarose gel. Fig. 1a and c show the



**Fig. 1** Cleavage of [ $^{125}$ I]-labeled Ad7 and Ad2 DNA with restriction enzymes. a-d. [ $^{125}$ I]-Ad7 DNA (1  $\mu$ g) was digested with BamHI (31) (a & b) and HpaI (20) (c & d). e-h. [ $^{125}$ I]-Ad2 DNA (4  $\mu$ g) was digested with SalI (23) (e & f) and BamHI (g, h). One unit of enzyme was added in each case and the incubation was for 1 h at 37°C. The DNA fragments were fractionated on a 1.4% agarose gel and the ethidium bromide fluorescence was photographed (20) (a, c, e and g). b, d, f and h represent the autoradiography of [ $^{125}$ I] labeled DNA fragments, fractionated on gels a, c, e and g, respectively.

cleavage pattern of BamHI and HpaI of [ $^{125}$ I] labeled Ad7 DNA, respectively. Fig. 1b and d show the autoradiography of the dried gel from Fig. 1a and c. Only the terminal fragments BamHI-F and J and HpaI-A and C (21, our unpublished observations) were labeled. Fig. 1e and g show the cleavage pattern of SalI and BamHI of [ $^{125}$ I] labeled Ad2 DNA and Fig. 1f and h show the autoradiography of the same gel. All the label was incorporated into the terminal fragments, SalI-A and B and BamHI-A and B (22,23). The internal SalI-D fragment which maps between 24.7 and 25.6 map units was too faint and is not seen in this gel. The results in Fig. 1 indicate that the label was specifically bound to terminal fragments of both Ad7 and Ad2 DNA.

Ad7 DNA-protein complex was isolated, labeled with [ $^{125}$ I] and subsequently treated with protease K. A protease resistant peptide containing a portion of the total [ $^{125}$ I] label still remained associated with the DNA and the restriction enzyme analysis demon-

strated that the label was also associated with the terminal fragments identical to the results shown in Fig. 1a-d. Moreover, the specific activity of the labeled DNA was approximately the same ( $3 \times 10^5$  cpm of [ $^{125}$ I] per  $\mu$ g of DNA) because the number of tyrosine residues that were susceptible to labeling and resistant to protease was the same whether labelling preceded or followed protease treatment. We do not know, in the present studies, exactly how much of the 55K terminal protein is resistant to protease, since the complex purified by velocity sedimentation on sucrose gradients containing guanidine hydrochloride was found to contain a number of other virion proteins associated with the terminal 55K protein (24). These data indicate that the iodlatable moiety at the termini of Ad DNA probably originates from the terminal protein.

The linkage between DNA and [ $^{125}$ I] labeled moiety was stable to treatment with 4M hydroxylamine, pH 4.2, at 37°C for 2 h. Under these conditions hydroxylamine is known to cleave phosphamide bonds while phosphodiester bonds are hydroxylamine-resistant (25). On the other hand, over 80% of the label was rendered acid-soluble after incubation with 0.1 N NaOH at 37°C for 17 h (Fig. 2). This observation can serve as an indication for presence of a phosphodiester bond between the terminal phosphate and a hydroxyl group of serine, threonine, or probably also tyrosine since these bonds are particularly sensitive to

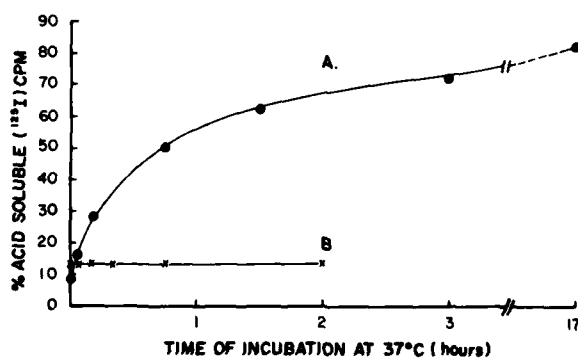
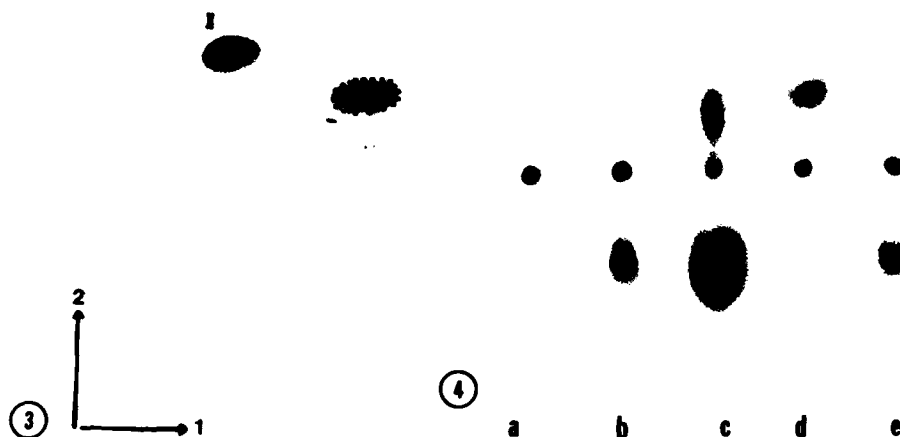


Fig. 2 Effects of alkali (—●—●—) and hydroxylamine (—X—X—) treatments on the linkage of [ $^{125}$ I] tyrosine-peptides to Ad7 DNA. [ $^{125}$ I] labeled Ad7 DNA (1.5  $\mu$ g) was incubated with 0.1 N NaOH or with 4M hydroxylamine at pH 4.2 at 37°C. The aliquots (2.5  $\mu$ l) were removed at indicated intervals of time and the acid-soluble radioactivity was measured (32) using a Packard gamma counter. The background for acid-soluble cpm was 10-13% due to the presence of [ $^{125}$ I] incompletely removed by agarose exclusion chromatography.



**Fig. 3** Two-dimensional thin layer chromatography.  $[^{125}\text{I}]$  labeled Ad7 DNA was hydrolyzed in the presence of 2N HCl as described in the text. The hydrolysate was chromatographed in two dimensions in cellulose-coated thin layer plate as described (26). The position of unlabeled moniodotyrosine marker was determined by ninhydrin staining and is indicated by the dashed outline. The relative amount of spot X was variable, increasing with the time of acid hydrolysis. In this experiment, the moniodotyrosine spot contained 37% of the input.

**Fig. 4** Ionophoretic separation at pH 1.9 of  $[^{125}\text{I}]$  labeled Ad7 DNA. Ad7 DNA was extracted from the virion after proteinase K treatment and labeled with  $[^{125}\text{I}]$  as described in the text. a. DNaseI (Worthington Biochemical Corp.) digestion of  $[^{125}\text{I}]$  Ad7 DNA. b. DNaseI, followed by snake venom phosphodiesterase. c. Denatured  $[^{125}\text{I}]$ -Ad7 DNA (7.8  $\mu\text{g}$ ) after treatment with  $\text{S}_1$  nuclease. d. Micrococcal nuclease digestion of  $[^{125}\text{I}]$ -Ad7 DNA. The reaction was terminated by heating to  $90^\circ\text{C}$  for 5 min. One half of the sample was treated with *E. coli* alkaline phosphatase (1 unit) at  $37^\circ\text{C}$  for 1 h and the sample was then applied to Whatman 3MM paper (e). Electrophoresis was carried out using formic/acetic acid buffer (pH 1.9) system (19) at 2400 volts. At the end of electrophoresis, the paper was dried and autoradiographed.

alkali treatment (25). This result is consistent with the observation of Carusi (9) who showed that alkali treatment of the viral DNA permitted labeling of the 5' end by alkaline phosphatase, polynucleotide kinase and  $[\gamma\text{-}^{32}\text{P}]$  ATP.  $[^{125}\text{I}]$  labeled DNA was acid hydrolyzed and analyzed by two dimensional thin layer chromatography. Autoradiography (Fig. 3) revealed two spots. One of these spots comigrated with the moniodotyrosine standard and the other (spot X) migrated as free  $[^{125}\text{I}]^-$  control (not shown).  $[^{125}\text{I}]^-$  or  $[^{125}\text{I}]\text{Cl}$  appeared due to partial decomposition of moniodotyrosine during acid hydrolysis (16). Prior to acid hydrolysis, the material contained no free moniodotyrosine and a small amount of unbound  $[^{125}\text{I}]$  (data not shown). These results indicate that the  $[^{125}\text{I}]$  label was incorporated into a tyrosine residue(s) present in the terminal peptide.

In order to further investigate the nature of the linkage between the [ $^{125}\text{I}$ ]-tyrosine-containing moiety and Ad7 DNA, the labeled DNA was digested either with DNaseI and snake venom phosphodiesterase or denatured and subsequently treated with  $S_1$  nuclease. The products were analyzed by paper electrophoresis at pH 1.9. Fig. 4 (a-c) shows the autoradiogram of such an experiment. The DNaseI digestion generated a series of oligonucleotides bound to the labeled moiety which moved towards the anode (Fig. 4a). However, when the DNaseI digest was treated with snake venom phosphodiesterase, a 3'  $\rightarrow$  5' exonuclease, the mobility towards the anode was reversed, and the radioactivity migrated towards the cathode (Fig. 4b). When the denatured [ $^{125}\text{I}$ ]-Ad7 DNA was treated with  $S_1$  nuclease (Fig. 4c), material migrating towards the cathode was produced, indicating that the linkage between the labeled moiety and the terminal nucleotide is also sensitive to  $S_1$  nuclease. The most plausible explanation of these results is that the terminal peptide is linked to the 5'-terminal nucleotide via a phosphodiester bond and venom phosphodiesterase and  $S_1$  nuclease cut the bond between the peptide and the 5' phosphate.

In order to confirm this explanation, the labeled DNA was treated with micrococcal nuclease, which cleaves the DNA, generating 3'-phosphate and 5'-OH terminated oligonucleotides. The [ $^{125}\text{I}$ ] labeled moiety which migrated towards the anode was produced (Fig. 4d). When this material was subsequently treated with *E. coli* alkaline phosphatase, it migrated towards the cathode as in the case of venom phosphodiesterase or  $S_1$  treatments (Fig. 4e). This observation can be explained if we assume that micrococcal nuclease generates peptides linked to a terminal phosphate moiety which originally represented the 5' terminal phosphate of DNA. This phosphate was removed by phosphatase treatment. Identical results were obtained when [ $^{125}\text{I}$ ]-Ad2 DNA was used in these studies. The data presented above indicate that tyrosine-containing peptide moiety is bound to the 5' ends of Ad DNA most likely through a phosphodiester bond. Ambros and Baltimore and Rothberg et al. have shown that the 5' end of poliovirus RNA is linked to the terminal VPg protein via a phosphodiester bond which is formed by the hydroxyl group of a tyrosine residue (26,27). In Ad system, we do not yet have any evidence indicating which amino acid residue participates in the formation of the linkage. The tyrosine residue which was iodlatable is probably not linked to the terminal phosphate of Ad DNA. It was reported

that a free hydroxyl group is necessary for the incorporation of I atom to the phenyl ring of tyrosine (27,28). Further experiments are in progress to identify the amino acid that is linked to the terminal nucleotide of Ad DNA. We are also investigating whether iodination of the terminal peptide of Ad7 and Ad2 can be extended to Ad DNA of other groups. This new method of terminal labeling can be useful in physical mapping of Ad DNA molecules with several restriction endonucleases. The fact that two Ad DNAs belonging to different groups sharing less than 15% homology (29) have their nucleic acids linked to the terminal protein by the same type of linkage is consistent with the results of Green et al. (30) who have shown a remarkable similarity in the peptide maps of Ad2 and Ad7 terminal proteins. Future research will throw light on the biological function of the terminal protein.

#### ACKNOWLEDGEMENTS

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## Comparative sequence analysis of the inverted terminal repetitions from different adenoviruses

(length of repetition/correlation with oncogenicity/conserved homologous sequences)

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**ABSTRACT** The comparative nucleotide sequences of the region of inverted terminal repetition from a representative member of group C (nononcogenic), group B (weakly oncogenic), and group A (highly oncogenic) adenoviruses are analyzed. Our data show that (i) the length of this unique region increases with the oncogenicity of the serotype, (ii) a unique homologous region—14 nucleotides long—is present exactly at the same distance from the terminus, and (iii) a hexanucleotide sequence, T-G-A-C-G-T, is present at the site where the terminal repetition diverges.

Human adenovirus (Ad) isolates have been grouped on the basis of immunological properties, mainly because of antigenic determinants on hexon, fiber, and penton base (1-3). There are 31 well-recognized human Ad serotypes. Some human Ad serotypes share several biological and structural properties, and attempts have been made to arrange the serotypes into groups. One of the most interesting group arrangements is based on the different oncogenic properties of the Ads (4-6). Green *et al.* (7) have reported the existence of five distinct DNA homology groups, A-E, which are consistent with the classification based on tumorigenicity in newborn hamsters.

Human Ads contain linear double-stranded DNA molecules with an estimated molecular weight of  $20-25 \times 10^6$  (8, 9). Ad DNA possesses a unique inverted terminal repetition (ITR) of the type  $a'b'c' \dots cba$ , which permits the formation of single-stranded circular DNA after denaturation and rehybridization (10, 11). A protein with a molecular weight of 55,000 is covalently linked to each 5' terminus of Ad2 and Ad5 DNAs (12-14). The terminal sequences presumably are biologically important because the origin and termination of DNA replication are in these regions (15-21). The exact mechanism by which the ITR is involved in replication still is unknown, although a model has been proposed (21, 22). There is evidence that the initiation of DNA replication occurs within the terminal 75 base pairs (17).

The sequences of ITR of Ad2 and Ad5 have been determined and shown to be 103 base pairs in length (23, 24), although a variant of Ad2 in which an ITR of 102 base pairs also has been reported (25). We studied the sequences of the ITR of Ads from different serotypes in order (i) to examine the correlation, if any, between the nucleotide sequence and the oncogenic property of a particular serotype, (ii) to probe into the biological role of this unique region, and (iii) to localize any homologous sequences, if any, that might serve as recognition signals for host enzymes involved in DNA replication. We report the complete sequence of the ITRs of Ad7 (group B) and Ad12 (group A). The ITRs of Ad7 and Ad12 are 136 and 162 base pairs in length,

respectively. There is an interesting correlation between the length of the ITR and the oncogenic properties of the Ad. In addition to a 14-nucleotide homologous sequence present in all Ad serotypes so far studied, we find a hexanucleotide sequence present at the site where ITR is terminated.

### MATERIALS AND METHODS

**DNA.** Ad7 (Greider) and Ad12 (Huie) were grown in KB cells, the virions were purified, and the DNA was extracted as described (26).

**3'-End Group Analysis.** The 3' ends of Ad7 and Ad12 DNAs were identified as described (27).

**5'-End Group Analysis.** The 5' ends of Ad2, Ad7, and Ad12 were deblocked by alkali treatment and labeled by using polynucleotide kinase and [ $\gamma$ - $^{32}$ P]ATP as described (28). The 5'-labeled DNA was cleaved with *Hha* I, which gave rise to a single terminal fragment due to a recognition site within the ITR in Ad2, Ad7, and Ad12 DNA. The labeled DNA was eluted and the 5'-end group analysis was carried out as described (29).

**DNA Sequence Analysis.** The 3' termini of Ad7 and Ad12 were labeled by using [ $\alpha$ - $^{32}$ P]dGTP (Amersham; specific activity 2000-3000 Ci/mmol; 1 Ci =  $3.7 \times 10^{10}$  becquerels) and T4 DNA polymerase (30). The 3'-labeled DNA was cleaved with *Bam*HI (31) for Ad7 and *Eco*RI for Ad12. The digests were fractionated by electrophoresis on 1.4% (wt/vol) agarose gel. The labeled terminal fragments were detected by autoradiography and were eluted from the gel as described (32). Alternatively, the 5' termini of Ad7 and Ad12 DNAs were labeled as described above. The labeled terminal fragments, *Bam*HI F and J of Ad7 and *Eco*RI A and C of Ad12 DNA (see Fig. 1) were subjected to sequence analysis according to Maxam and Gilbert (33). The 5'-labeled *Hha* I fragment of Ad12 was digested partially with DNase I and then with snake venom phosphodiesterase as described (34). The partial digest was fractionated by a two-dimensional system (34-36).

### RESULTS

We compared the restriction enzyme cleavage patterns of Ad2, Ad7, and Ad12 in order to establish the identity of the strains used by us. Fig. 1 shows the cleavage patterns of Ad2, Ad7, and Ad12 DNAs with *Eco*RI and *Bam*HI. The cleavage patterns of Ad2 DNA agreed well with those in the literature (37). The Ad7 (Greider) strain that we used is identical to the one for

Abbreviations: Ad(s), adenovirus(es); ITR, inverted terminal repetition.

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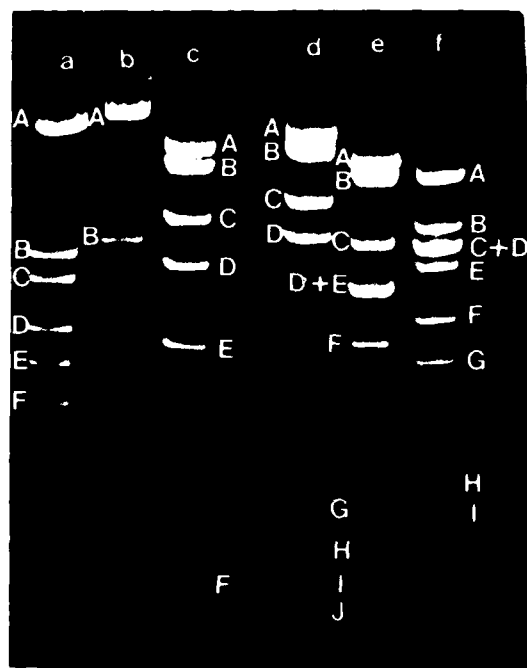


FIG. 1. Restriction enzyme cleavage patterns of Ad2, Ad7, and Ad12 DNA. The DNA fragments were fractionated on 1.4% (wt/vol) agarose gel and stained with ethidium bromide. Lanes: a, Ad2/*EcoRI*; b, Ad7/*EcoRI*; c, Ad12/*EcoRI*; d, Ad2/*BamHI*; e, Ad7/*BamHI*; f, Ad12/*BamHI*.

which *EcoRI* and *HindIII* maps have been established (38). Based on the cleavage patterns of Ad7 (Greider) observed with *Sal I*, *Hpa I*, *Sma I*, *HindIII*, and *BamHI*, the strain was classified as the Ad7a subtype, which has been shown to be characteristically different from the Ad7 (Gomen) prototype strain both from immunological properties (39) and from restriction enzyme analysis (40). However, *EcoRI* cleavage of Ad7 (Greider) gave rise to only two fragments in contrast to cleavage of the Ad7a subtype (38). Ad12 DNA gave rise to identical patterns reported elsewhere for this strain (41, 42).

The 3'-end group analysis (27) of Ad2 DNA has shown a dG residue (25). Using this method, we found that the 3' termini of Ad7 and Ad12 DNAs also consisted of dG residues (data not shown). The 5'-terminal nucleotide of Ad2, Ad7, and Ad12 determined subsequent to deblocking the termini (28) was found to be the complementary dC residue (Fig. 2, lanes a-c). These data suggested that the termini of Ad7 and Ad12 DNAs are flush, similar to those in Ad2 (25) and Ad5 (23).

Fig. 3 shows the sequencing gels of Ad7 (a and b) and Ad12 (c and d) at the ITR that enabled us to deduce the complete sequence and the length of this unique region. Sequences of this region in Ad2 (24, 25), Ad7, and Ad12 are shown in Fig. 4. The sequences from the two ends start to diverge at nucleotide 137 from the ends of Ad7 and at nucleotide 163 from the ends of Ad12 (shown by arrows). Sequence analysis carried out from the labeled 5' ends of Ad7 and Ad12 DNA (data not shown) confirmed our sequence data obtained from the 3' ends.

## DISCUSSION

Comparative sequence analysis of the ITR of three different serotypes—Ad2, Ad7, and Ad12—shows a remarkably conserved sequence of 14 nucleotides, first noted by Tolun *et al.* (44) in Ad3, in simian Ad7, and in a partial sequence at the ITR of Ad12. Our sequence data for the ITR of Ad12 at nucleotides

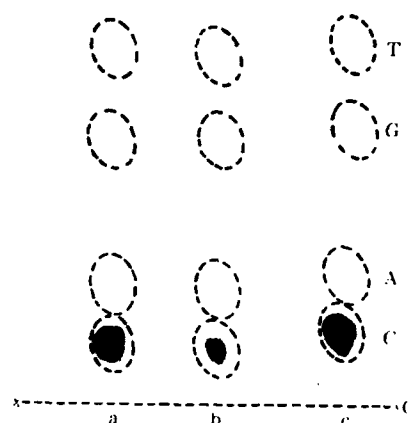


FIG. 2. 5'-End group analysis of Ad2, Ad7, and Ad12 DNA. Viral DNA was labeled at its 5' termini as described (28). The 5'-end group analysis was carried out as described (29), except that the 5'-mononucleotides were separated by electrophoresis at pH 3.5 (43). O, origin; dashed outlines, positions to which the unlabeled 5'-mononucleotide markers migrated in this system. Lanes a, b, and c represent the 5'-end group analysis carried out on Ad2, Ad7, and Ad12 DNA, respectively.

6-8 (T-C-A) (Fig. 4) differ from those of Tolun *et al.* (T-A-T) (44). This difference is probably attributable to the two strains studied.

Although the function of this 14-nucleotide homology is not known, its appearance at a constant distance from each terminus suggests that it may serve as a recognition signal, possibly for a host enzyme. In addition, we determined the complete sequence at the ITR of human Ad7 and Ad12. The length of the ITR is directly proportional to its oncogenic potential. It is interesting to note that the length of the ITR is identical (or almost) for two representative members of the same group—e.g., Ad2 and Ad5, or Ad3 and Ad7.

We find an additional interesting homology in the comparative sequence data in Fig. 4. A hexanucleotide sequence, T-G-A-C-G-T, is present in Ad2, Ad3, Ad5, Ad7, and Ad12 at or near the site where the sequences beyond the ITR begin to diverge. Other homologous regions present in three different serotypes are shown within the boxes. Specific nucleotide substitutions that occurred in Ad serotypes within a group (such as Ad2 and Ad5, Ad3 and Ad7) are outlined (Fig. 4). It is interesting to note that there are no mutations within this unique homologous region of 14 nucleotides.

The molecular mechanism of replication of Ad DNA is currently under study in several laboratories. The nascent daughter strands in replicative intermediates are heterogeneous in length but always are shorter than the unit length of mature Ad2 DNA (45). The synthesis of both daughter strands terminates at or near their 3' ends (15, 18). All available data support a model proposed for DNA replication in which the synthesis is initiated at or near either end of the double-stranded Ad DNA (15-22, 46). Following initiation, daughter-strand synthesis proceeds in the 5'-to-3' direction with the simultaneous displacement of the parental strand with the same polarity. Initiations at the left and right molecular ends are approximately equal in frequency, and multiple initiations on the same replicating molecule are common (21). At any given displacement fork, only one of the two parental strands is replicated. The displaced strand is presumably replicated later, after completion of the displacement synthesis (21, 47).

Little is known regarding the mechanism of initiation of

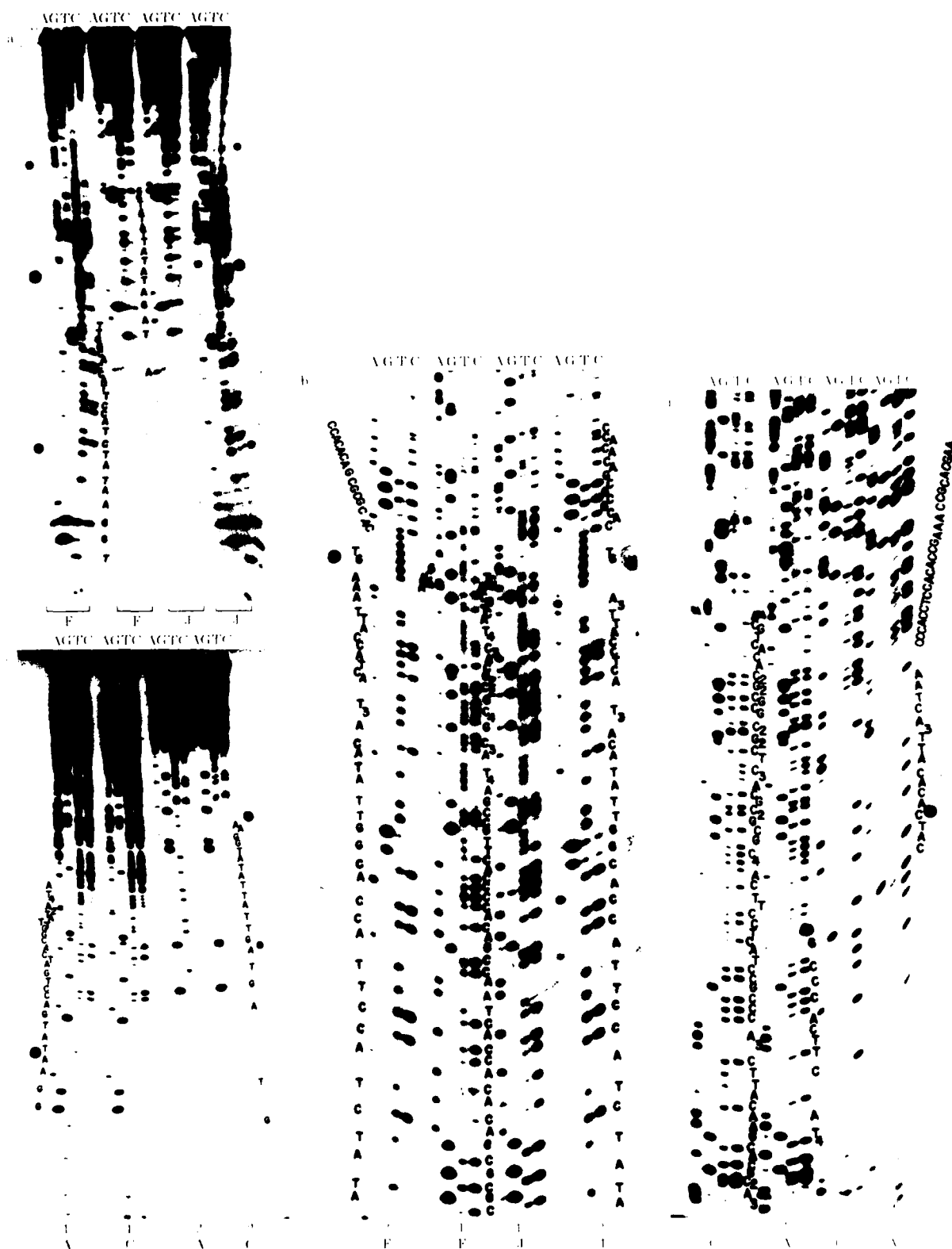


FIG. 3. Autoradiographs of the sequence gels. (a and b) Ad7 DNA was labeled at the 3' termini by using T4 DNA polymerase and [ $\alpha$ - $^{32}$ P]dGTP. The labeled DNA was digested with *Bam*HI, and the terminal *Bam*HI F and J fragments (see ref. 40) were purified and subjected to DNA sequence analysis (33). (c and d) The 3'-labeled Ad12 DNA was digested with *Eco*RI and the purified terminal *Eco*RI A and C fragments were used for sequence analysis. A 25% (wt/vol) gel (40  $\times$  20  $\times$  0.075 cm) was used (a and c) to fractionate 1-50 nucleotides from the labeled terminus and a 10% (wt/vol) gel (80  $\times$  25  $\times$  0.075 cm) was used (b and d) to fractionate 40-190 nucleotides. Each set of samples was loaded twice (indicated by numbers). The arrow in b and d indicates the point at which the ITR is terminated.

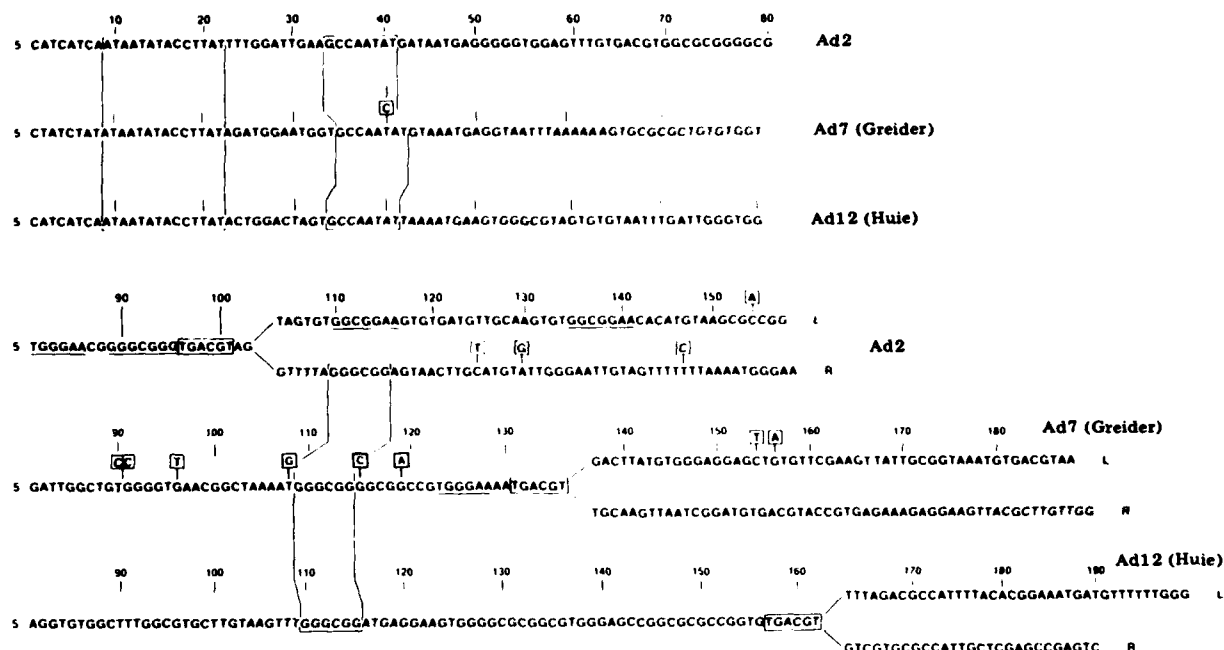


FIG. 4. Sequences of the ITR of Ad2, Ad7, and Ad12 DNA. Sequence data of Ad2, Ad3, and Ad5 DNA have been published (23-25, 44). The boxed nucleotides indicated at specific locations at the top of sequence data for Ad2 and Ad7 represent the nucleotide substitutions found in the closely related Ad5 and Ad3, respectively. The sequences homologous among Ad2, Ad7, and Ad12 are enclosed in a box. The hexanucleotide sequence T-G-A-C-G-T at the vicinity where ITR is terminated is underlined.

DNA replication at (or near) the termini of duplex Ad DNA or at the terminus of the displaced parental strand. The presence of identical sequences at the termini suggests that a single mechanism may be involved for all initiation events during Ad DNA replication. Initiation at the 3' terminus of a displaced parental strand could proceed through circular intermediates formed by hybridization of the self-complementary terminal sequences (21, 22). Although it seems likely that initiation of Ad replication takes place in the region of the ITR, the exact location of the origin of replication has not been determined experimentally. If the ITR is involved in the replication of the displaced strand through the mechanism proposed in the model, then it is not clear why group B (Ad3, Ad7) and group A (Ad12) have longer ITR than do Ad2 or Ad5, although all Ads replicate in the same host.

Challberg and Kelly (48) described a soluble enzyme system from Ad-infected cells that was capable of replicating exogenously added Ad2 or Ad5 DNA (in the form of a complex with the terminal protein). It is not known at present whether the extract prepared from group C Ad-infected cells can replicate group B Ad (Ad7) or group A Ad (Ad12) DNA *in vitro*. Future studies will unravel the biological role of this unique region of ITR found in several Ad serotypes.

#### ADDENDUM

After submission of this paper, we noticed a recent report by Dijkema and Dekker (49) that described the sequence analysis of the ITR of Ad7 prototype (Gomen) strain. Comparison of their data on Gomen strain with ours on Greider strain reveals that (i) the length of ITR is preserved in these two strains, (ii) the sequence at the ITR of Gomen strain differs from the Greider strain at nucleotides 3 (C), 40 (C), 99 (T), 101 (A), and 106 (C) (Fig. 4), and (iii) the homologous sequences located at nucleotides 9-22 and at the site where the ITR ends (T-G-A-C-G-T) are preserved. The two strains of Ad7 exhibited dif-

ferent cleavage patterns when analyzed by restriction enzymes *EcoRI*, *Hpa I*, *Sma I*, *HindIII*, *BamHI*, and *Kpn I* (unpublished results; and refs. 40 and 50).

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